

Mats Bjellerup

TETRACYCLINE PHOTOTOXICITY

An experimental and clinical study



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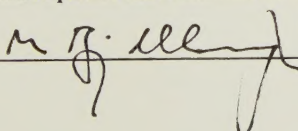


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Abstract The phototoxic potency of different tetracycline derivatives was investigated experimentally in two in vitro systems; (i) photolysis of erythrocytes (photohemolysis); (ii) growth inhibition in cultured human fibroblasts. Pronounced differences in phototoxic ability were found with the following order of potency; demethylchlortetracycline = chlortetracycline = doxycycline >> methacycline > tetracycline = oxytetracycline = lymecycline > minocycline. The correlation between the phototoxic ranking obtained by these two experimental techniques and clinical reports on photoreactions in humans undergoing tetracycline therapy thus appears to be good, indicating the predictive value of the photohemolysis and fibroblast growth inhibition techniques. In a double blind cross-over study in human volunteers the same order of relative phototoxic potential could be verified comparing doxycycline and lymecycline, using different modalities of artificial UVA (long-wave ultraviolet radiation) and recording objective as well as subjective reactions. No correlation was found between the administered dose of tetracycline derivative, serum level and phototoxic reactivity indicating the importance of other individual factors. UVB (medium-wave ultraviolet radiation) augmentation of the phototoxic reaction elicited by doxycycline and UVA was verified by investigations in mice registering inflammatory tail edema. Two types of UVB augmentation were detected; a general augmentative effect of UVB given either immediately before or after the phototoxic reaction and an enhanced augmentative effect of UVB given 24 h before the phototoxic reaction, possibly reflecting an increased concentration of doxycycline in inflamed tissue. The phenomenon of UVB-augmentation offers a possible explanation of present and previous difficulties in provoking phototoxic tetracycline reactions in humans using artificial UVA or filtered sunlight.		
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TETRACYCLINE PHOTOTOXICITY

An experimental and clinical study

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TETRACYCLINE PHOTOTOXICITY

An experimental and
clinical study

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Malmö 1986

To Anne,
Jesper and Jakob

LIST OF PAPERS

This thesis is based on the following papers referred to in the text by their Roman numerals.

- I. Bjellerup M, Ljunggren B. Studies on photohemolysis with special reference to demethylchlortetracycline. Acta Derm Venereol (Stockh) 1984;64:378-383
- II. Bjellerup M, Ljunggren B. Photohemolytic potency of tetracyclines. J Invest Dermatol 1985;84:262-264
- III. Bjellerup M, Kjellström T, Ljunggren B. Influence of tetracycline phototoxicity on the growth of cultured human fibroblasts. J Invest Dermatol 1985;85:573-574
- IV. Bjellerup M, Ljunggren B. Double blind cross-over studies on phototoxicity to three tetracycline derivatives in human volunteers. Submitted for publication.
- V. Bjellerup M. Medium-wave ultraviolet radiation (UVB) is important in doxycycline phototoxicity. Acta Derm Venereol, in press 1986

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1. ABBREVIATIONS

ACD	acid-citrat-dextrose
Chlor	chlortetracycline
CPZ	chlorpromazine
DMCT	demethylchlortetracycline
Doxy	doxycycline
EPR	electron paramagnetic resonance
Lyme	lymecycline
MED	minimal erythema dose
Metha	methacycline
Mino	minocycline
8-MOP	8-methoxypsoralen
Oxy	oxytetracycline
RBC	red blood cell
TC	tetracycline
Tetra	tetracycline HCl
UV	ultraviolet
UVA	long-wave ultraviolet radiation (320-400 nm)
UVB	middle-wave ultraviolet radiation (290-320 nm)
UVC	short-wave ultraviolet radiation (200-290 nm)
UVR	ultraviolet radiation

2. INTRODUCTION

A. PHOTODERMATOSES

Radiation emitted from the sun is essential for all forms of life on earth. It can be described in several ways one of which is as discrete units of energy, so called quanta. It could also be described as a wave system and a part of the electromagnetic radiation according to Maxwell's concept (1). In this system of waves of different wavelengths, radiation visible to the human eye as light occupies only a small part of the spectrum as shown in fig 1.

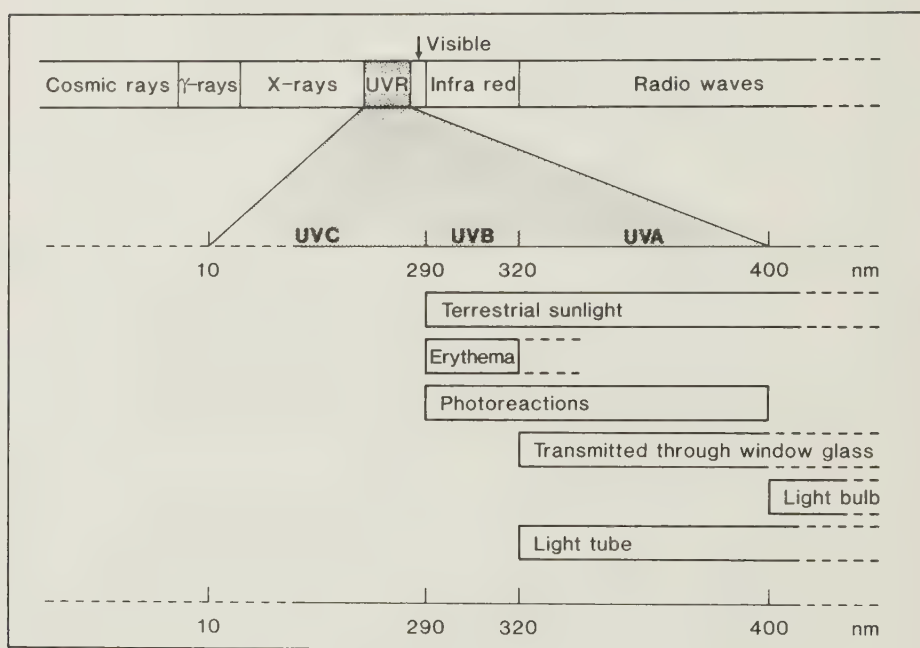


Fig 1. The upper bar illustrates the full electromagnetic spectrum with magnification of the UVR region divided into its components UVC, UVB and UVA. Other aspects of biological effects on man as well as some physical properties of window glass filters and artificial light sources are included in the lower bars.

The sun is a nuclear fusion reactor which derives its energy principally from the fusion of hydrogen atoms into helium with concomitant production of energy, partly in the form of heat but mainly in the form

of electromagnetic radiation containing extremely short waves, gamma rays, which on their way to the solar surface are transformed into longer wavelengths (2). Solar radiation reaches the earth's surface with a maximum near the middle of the visible region; 40% of the total radiation is in the visible, 51% in the infra-red and 9% in the ultra-violet region (3). The different components of solar radiation, focusing on ultra-violet radiation, are shown in fig 1.

Different parts of solar radiation, natural as well artificial, have profound biological effects on man, some of which are beneficial and some harmful. Among the beneficial effects may be mentioned regulation of bio rhythms such as sleep, body temperature and secretion of hormones, UVR-stimulation of vitamin D₃ synthesis, increased psychological well being and resistance to infections, beneficial effects on certain skin diseases such as psoriasis, acne and atopic eczema (4).

Under certain conditions and in certain individuals, however, sunlight induces harmful effects in human skin as is the case with the photodermatoses i.e. skin diseases caused by normal reactions to excessive doses of solar radiation or pathological reactions to normal doses of sunlight. These reactions are mainly produced by the UV-part of the solar spectrum and can be roughly classified in the following subgroups which will be briefly described:

- Phototrauma
- Chemical photosensitization
- Dermatoses affected by sunlight
- Polymorphic light eruption
- Solar urticaria

PHOTOTRAUMA. In this group are included both acute and chronic reactions. The acute reaction manifests itself as sunburn which in its mildest form consists of only erythema but in its severe form also as edema and blistering. The most effective part of the solar spectrum in producing this effect is the region around 300 nm (1). The chronic reactions manifest themselves as; (i) ageing of the skin with epidermal atrophy and dermal elastosis, (ii) development of skin cancer especially in the form of spinocellular cancer. The most effective part of spectrum inducing chronic reactions seems to be in the UVB possibly with some contribution also from the UVA (4).

CHEMICAL PHOTSENSITIZATION. Certain exogenous or endogenous chemicals in the skin are able to absorb radiation at a wavelength specified for each substance. The energy thus absorbed enables the activated molecule to cause biological damage through direct or indirect mechanisms. The wavelength(s) causing biological damage i.e. the action spectrum for the chemical is most frequently in the UVA but with some substances in the UVB (5). Two types of clinical reactions can be elicited in this way, namely phototoxic and photoallergic reactions which will be described later.

DERMATOSES AFFECTED BY SUNLIGHT. Some dermatoses not primarily caused by sunlight may be triggered off or made worse by solar radiation as is the case with herpes simplex infections and cutaneous lupus erythematosus. Even psoriasis, which in the majority of cases benefits from sun exposure, may in some cases be worsened.

POLYMORPHIC LIGHT ERUPTION. This disease is very common if milder cases are included. After a latency period of some hours to several days after sun exposure an itching eruption develops on exposed skin. As indicated by its name this eruption may take several clinical forms in different persons. The underlying mechanism(s) is not known though an immunological reaction has been proposed. In recent years repeated, large doses of artificial UVA have been shown to provoke the reaction in a majority of patients (6).

SOLAR URTICARIA. This rare disease evolves during or within a few minutes after exposure to sunlight and has been provoked artificially with different parts of the solar spectrum (1).

B. CHEMICAL PHOTSENSITIZATION

The first one to clarify that chemical photosensitization could express itself in two different ways was Stephan Epstein in 1939 (7). Influenced by several reports on skin reactions to sulphanilamide and a possible correlation with sunlight exposure he carried out a series of experiments with intradermal injections of this drug followed by natural and artificial UV-irradiation. Performing this he noted in all 6 test persons an erythematous, non pruritic reaction occurring after the first UV-exposure

and within 24 hours. This reaction was only quantitatively different from ordinary ultraviolet erythema and the phenomenon was termed primary photosensitivity. In 2 out of the same 6 test persons an entirely different picture emerged consisting of an urticarial inflammatory, pruritic reaction developing 10 days after the primary reaction and at subsequent tests with a shorter incubation period. This reaction was morphologically entirely different from ordinary ultraviolet erythema and this phenomenon was termed photoallergy. The same classification holds true today though the term primary photosensitivity has now been replaced with phototoxicity.

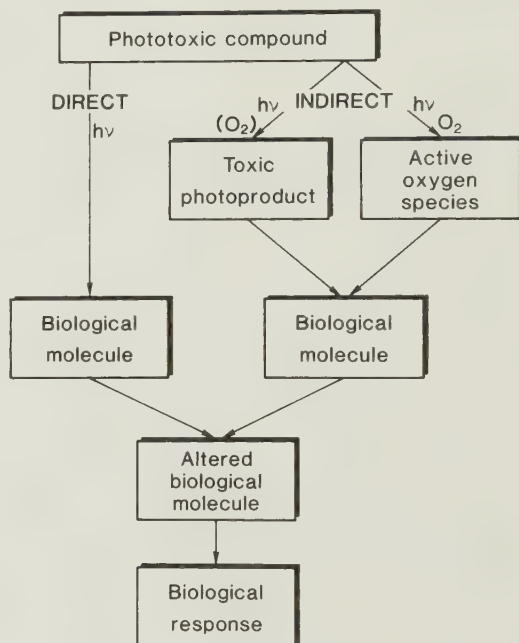
PHOTOTOXICITY. This type of reaction per definition can be provoked in all individuals provided that the concentration of the phototoxic chemical and the irradiation dose of the appropriate wavelength delivered to the target organ are sufficiently large. Such reactions in man can be provoked with internal as well as external chemicals (8). Among internal chemicals should be mentioned sulphonamides, phenothiazines, benzodiazepines, tetracyclines, nonsteroidal antiinflammatory drugs, amiodarone and nalidixic acid. Among external phototoxic chemicals applied to the skin intentionally or by accident, the psoralens are the most important.

The phototoxic process can be divided into two steps (9). The first step includes light absorption by the chemical (photosensitizer) and the resulting chemical reactions involving biological molecules. These reactions need not involve only one type of biological molecule since with many photosensitizers it has been shown that many different types of biological molecules such as DNA, RNA, cell membranes and soluble proteins are altered chemically by one and the same photosensitizer. The second step in the phototoxic process involves the biological response to the chemical reactions taking place in the first step and can be recorded for example as lysis of cells, cell growth inhibition, skin erythema and sensation of pain.

Returning to the first step of photosensitized damage to biological molecules, it can be divided into two categories (9); (i) direct mechanisms where the excited phototoxic compound reacts directly with the biological substrate by means of addition to a biological molecule, fragmentation to form unstable radicals subsequently reacting with the biological substrate or photoionization producing cation radicals and

electrons which react with the biological molecule; (ii) indirect mechanisms where another molecule (a photoproduct of the phototoxic compound or an activated oxygen species) reacts with the biological substrate. The toxic photoproduct may or may not require oxygen for its formation; the active oxygen species per definition are derived from oxygen and exist as singlet oxygen, superoxide radical or hydroxy radical. The different pathways of the phototoxic reaction are illustrated in fig. 2.

Fig 2. Schematic illustration of the two steps of the phototoxic process, the first step resulting in an altered biological molecule, the second resulting in a biological response. The first step can be achieved by direct and indirect pathways.



PHOTOALLERGY. This type of reaction, apart from the photoactive chemical combined with radiation of suitable wavelength, requires in addition an activation of the immune system. It has been reported with internally as well as externally administered chemicals, the latter event being the more frequent (10). Among internally administered chemicals can be mentioned sulfonamides, chlorothiazide diuretics, sulfonylurea antidiabetic agents and quinidine. Several externally applied chemicals can induce photoallergic reactions including halogenated salicylanilides, musk ambrette, hexachlorophene and sunscreening agents.

The pathogenetic mechanism responsible for photoallergic responses to

chemicals is believed to involve interaction between the photoactive compound and skin proteins (11). Under the influence of radiation of the appropriate wavelength, the chemical and protein become covalently bound to one another. This photoadduct acts as an antigen inducing an immunological reaction in certain individuals inducing cell-mediated allergy or development of circulating antibodies.

C. TETRACYCLINES

HISTORY. The development of the tetracyclines was initiated by the therapeutic success of penicillin and streptomycin and was the result of a systematic screening of soil from many parts of the world for antibiotic producing microorganisms (12). The first one, chlortetracycline, was introduced in 1948, followed by oxytetracycline in 1950. Chlortetracycline is elaborated by streptomyces aureofaciens, oxytetracycline by streptomyces rimosus. A third member of this family, tetracycline, was introduced in 1952 when the chemical structure of these agents had been elucidated and is produced semisynthetically from chlortetracycline. Demethylchlortetracycline (DMCT), belonging to a new family of tetracyclines characterized by the absence of the ring-attached CH_3 group present in the others, was introduced in 1959 and is produced by a mutant of streptomyces aureofaciens. Methacycline, a semisynthetic derivative of oxytetracycline, was introduced in 1961. Later during the sixties three other derivatives became available: lymecycline, doxycycline and minocycline. Lymecycline is the synonym for tetracycline substituted with methylene lysine and acts as a prodrug dissociating in the gastrointestinal tract to yield tetracycline as has been recently shown (13). Doxycycline was made available for clinical use in 1966 and is produced by hydrogenation of methacycline. Minocycline, not registered in Sweden, has a dimethylamino group attached to the D-ring, lacking in other derivatives. For detailed chemical structures see fig 3.

ACTION. The tetracyclines possess a wide range of antimicrobial activity against gram-positive and gram-negative bacteria especially Mycoplasma, Chlamydia, Yersinia enterocolitica, most Escherichia coli, Proteus morganii, many Klebsiella, Serratia, Enterobactae, Citrobactae, most Hemophilus influenzae, most beta-hemolytic streptococci and many Bacteroi-

DERIVATIVE	INTRODUCTION	STRUCTURE
Chlortetracycline	1948	
Oxytetracycline	1950	
Tetracycline	1952	
Demethyl-chlortetracycline	1959	
Methacycline	1961	
Lymecycline	1964	
Doxycycline	1968	
Minocycline	~1965	

Fig 3. Chemical structures and generic names for the most important tetracycline derivatives, arranged according to their approximate date of introduction.

des (14). In vitro, these drugs are primarily bacteriostatic; in high concentrations they are frequently bactericidal. The sensitivity or resistance of a particular microorganism to each of the congeners is, with some exceptions, quite similar. The most probable mechanism of antimicrobial action is that of inhibition of protein synthesis. The tetracyclines bind specifically to 30 S ribosomes and thereby prevent access of aminoacyl t-RNA, an effect that can be reversed by washing, making it probable that the reversibly bound antibiotic is responsible for the antibacterial action (12).

PHARMACOLOGY. The tetracyclines are adequately but incompletely absorbed from the gastrointestinal tract, absorption being most active in the stomach and upper small intestine. The irregularity of their absorption from the gastrointestinal tract is responsible for the wide range of plasma levels in different individuals following oral administration. Peak plasma levels after a single dose of chlortetracycline are attained in 2 to 4 hours and persist for 6 hours or longer. For this reason the administration of 250 mg four times daily has been recommended for these derivatives producing plasma levels of 1-3 microg/ml (12). The later introduced derivatives are characterized by better absorption and longer half-life making the same plasma levels possible with lower doses administered at longer intervals, the extreme being doxycycline which is given in a dose of 100 mg once daily. Absorption is impaired by milk and milk products and particularly by aluminium hydroxide gels and calcium and magnesium salts.

The volume of distribution of the tetracyclines is relatively larger than that of the body water, indicating sequestration in some tissues. They are bound to plasma proteins in varying degree. All the tetracyclines are removed from the blood by the liver, where they are concentrated and then excreted, by way of the bile, into the intestine, from which they are partially reabsorbed (12). They diffuse into brain, spinal fluid, saliva, synovial fluid, pleural fluid, semen, prostatic fluid, placenta, fetal tissues and milk of lactating patients.

All the tetracyclines are excreted in the urine and the feces, the primary route being the kidney by glomerular filtration. With methacycline, DMCT and doxycycline approximately 50% is excreted in active form in the urine and 5% in the feces, the rest in non active form bound to chelating

agents in the feces.

CUTANEOUS PHARMACOLOGY. Simultaneous determinations of serum and skin levels of tetracycline and DMCT have been performed in humans using a spectrophotofluorometric method (15). In no case was either antibiotic detected in normal skin even in the presence of circulating drug. In areas of dermatitis of different types (including sunburn) however significant amounts of these antibiotics were detected. In patients with psoriasis, significant amounts were also found in uninvolved skin, only slightly less than in clinical lesions. The bulk of the measurable tetracycline was contained in the dermis. These results concur with those studies that demonstrate concentration of tetracycline in rapidly growing, necrotic or inflamed tissues.

D. TETRACYCLINE PHOTOTOXICITY

CLINICAL CONSIDERATIONS

HISTORY. The first case of tetracycline phototoxicity, to the author's knowledge, was reported in May 1949 when a paper by Kierland, Herrell and O'Leary, concerning treatment of syphilis with chlortetracycline administered by mouth, was read before the Sixty-Ninth Annual Meeting of the American Dermatological Association (16). They treated their patients with 2 to 4 g daily for up to 1 month and mention (in a separate discussion) one patient with acquired photosensitivity in the form of erythema solare persisting for a month after discontinuation of therapy. In June the same year the first case of photoonycholysis was reported, although not recognized as such, when solar dermatitis occurred in 2 patients and subungual hemorrhage in 1 patient out of 135 treated with chlortetracycline 2.5 g daily for brucellosis (17).

CLINICAL MANIFESTATIONS. The phototoxic skin changes with tetracyclines resemble those of minor to severe sunburn with erythema, edema and sometimes papules or blisters. The occurrence of paraesthesias, primarily tingling of the hands, feet and nose, is considered an early indicator of abnormal sunburn reaction (18). The phototoxic rather than photoallergic nature of the reaction is indicated by the following facts (19); (i) the

drug causes light sensitivity within the first day of treatment; (ii) the clinical picture is mostly a strong but morphologically normal sunburn reaction; (iii) the incidence of photosensitivity is dose dependent.

Photoonycholysis is usually combined with cutaneous reactions and involves the distal third of the nails sparing the lateral margins (20). Sometimes the patient complains of onychodynia although in most cases the complaint is of cosmetic nature only. In a couple of months after medication is discontinued there is complete restitution (20).

A clinical different picture with cutaneous changes consisting of fragility expressed as blistering, denudation after mild trauma and scar formation primarily involving the dorsa of the hands, clinically indistinguishable from skin changes noted in porphyric disorders, has been described in five individuals taking tetracycline hydrochloride and was considered to be a manifestation of a low-grade photosensitization (21).

FREQUENCY. The frequency of phototoxic reactions in the clinical situation with tetracycline derivatives shows great variations depending on several factors such as dosage, duration of treatment, age of patients, precautions to avoid sun exposure and what derivative has been given. The frequency of reactions can be calculated only with chlortetracycline, DMCT and doxycycline which have been reported in a large enough number of cases and varies between 1% and 31% in different reports (16, 17, 20, 22, 23, 24, 25, 26, 27, 29). With the other derivatives there are only scattered clinical reports and no calculations concerning frequency can be made.

RELATIVE PHOTOTOXIC POTENTIAL. Regarding all available reports on tetracycline phototoxicity there appear to be differences in the phototoxic potential of the different tetracycline derivatives according to table 1. With chlortetracycline, the first derivative to be introduced, there are four reports allowing frequency calculations (16, 17, 22, 23), giving a frequency of 16% in the report, where standard doses of the drug were prescribed (23). In addition there are some scattered clinical reports with this drug (30). In contrast, with oxytetracycline and tetracycline, the second and third derivative respectively to be introduced there are only scattered reports (31, 32 and 33, 34, 35, 18 respectively). The introduction of the fourth derivative, DMCT, was immediately followed by several reports on photoreactions, five of these allowing frequency cal-

	Year	Author	Total	Reactors	%	Reference
CTC	1950	Kierland	7	1	14	16
	1950	Harris	135	3	2	17
	1950	Schaffer	13	4	31	22
	1959	Jirasec		2		30
	1959	Krauskopf		2		30
	1965	Verhagen	63	10	16	23
OTC	1963	Tromovitch		1		31
	1984	DIC, Sweden		1		32
TC	1963	Segal		1		33
	1966	Cullen		1		34
	1971	Frank		2		35
	1973	Verma			given in	18
DMCT	1960	Morris	57	1	2	24
	1960	Falk	27	4	15	25
	1960	Carey	2682	40	1	26
	1960	Fuhrman	70	7	10	27
	1961	Orentreich	108	27	25	20
	1962	de Veber		1		39
	1962	Hicks			given in	18
	1963	Buytendijk			given in	18
	1984	DIC, Sweden		3		32
MC	-					
Lyme	1984	DIC, Sweden		1		32
Doxy	1966	Huang	55	4	7	29
	1971	Frank		1		35
	1973	Zuehlke		1		36
	1981	Cavens		3		37
	1982	Jeanmougin		1		38
	1984	DIC, Sweden		14		32
Mino	1981	Kestel		1		40

Table 1. Clinical reports on tetracycline phototoxicity grouped for each derivatives (CTC chlortetracycline, OTC oxytetracycline, TC tetracycline MC methacycline, Lyme lymecycline, Doxy doxycycline, Mino minocycline). Year of report, author, total number of patients, number of reactors, frequency % and reference are included. DIC = Drug Information Committee, Sweden.

culations of 2, 15, 1, 10 and 25% respectively (24, 25, 26, 27, 20). With methacycline there are no reports on phototoxicity of the classical type to the author's knowledge. During long-term treatment however the appearance of pigmentation of light-exposed skin considered to be of phototoxic nature has been reported (28). Among the later derivatives doxycycline is the most often reported (29, 35, 36, 37, 38, 32), while lymecycline and minocycline have been reported in only one case each (32 and 40 respectively). The considerations put forward above concerning phototoxic potential should of course be judged with caution being retrospective and incomplete. With this reservation however they may reveal certain significant trends. The recent report from the Swedish Drug Information Committee covering photoreactions with tetracyclines during the period 1972-83 reveals 14 reactions with doxycycline, 3 with DMCT and 1 with lymecycline and oxytetracycline respectively (the percental share of the Swedish market, calculated as daily doses in 1983, were 71, 0.004, 16 and 3% respectively) (32).

EXPERIMENTAL INVESTIGATIONS

METHODS. Several experimental methods, in vitro as well as in vivo, have been used for studies on different aspects of tetracycline phototoxicity, namely;

PHOTOHEMOLYSIS: The pioneering work using this method was done by Blum (41) and Cook (42) and was further developed by Kahn in 1971 (43). Red blood cells (RBC) irradiated with UVR in the presence of a photosensitizing agent may hemolyze secondary to membrane damage. The concentration of hemoglobin in the supernatant is proportional to the injury inflicted. Since RBC are devoid of cell nuclei this method is thought to reflect membrane damage exclusively. The underlying mechanism behind the lysis of RBC is a photodegradation of biomembranes probably synonymous with light-induced oxidation of membrane components (44). These oxidative reactions may involve different components of the cell membrane such as phospholipids, cholesterol and amino acids which in the presence of radiation, molecular oxygen and appropriate sensitizer may undergo processes of lipid peroxidation and destruction of amino acid side chains (44). Another indirect mechanism leading to lysis of RBC is the production of a

toxic photoproduct as has been shown with preirradiation of chlorpromazine (45, 46). Previous investigations concerning the photohemolytic ability of tetracyclines have been somewhat contradictory. Thus Kahn and Fleischaker could not show hemolysis to occur with DMCT and tetracycline (43) while other workers have found DMCT and oxytetracycline to be photohemolytic (47, 48, 49).

EXPERIMENTAL ANIMALS: Stratigos and Magnus were able to show photosensitization with DMCT given intradermally, and to some extent intraperitoneally, to mice irradiated with different modalities of UVR and assessed for erythema by way of reading a "blueing" reaction obtained by injection of a colouring agent intraperitoneally (50). Ison and Blank studied whole body erythema in hairless mice given different photoactive compounds intraperitoneally followed by irradiation and found DMCT and tetracycline to be phototoxic (51). A quantitative method, measuring phototoxic tail edema in mice given the photosensitizer intraperitoneally or by mouth, was developed by Ljunggren and Möller and showed positive results with DMCT, doxycycline and lymecycline (52).

CELL CULTURE: Different types of mammalian cells in culture have been used in phototoxicity studies. Human amnion cells were used by Chang and Weinstein in 1964 testing DMCT, methacycline and tetracycline showing DMCT and to some degree methacycline to be phototoxic (53). Freeman et al (54) and Freeman (55) using Hep-2 cells originating from human laryngeal carcinoma, monkey kidney cells, rabbit kidney cells and rabbit skin fibroblasts testing DMCT and tetracycline showed the first one to be phototoxic. Morison et al investigating $[^3\text{H}]$ -thymidine incorporation into nuclear DNA of human peripheral blood mononuclear cells could demonstrate a reduced incorporation when cells were treated with DMCT and UVA (56).

STUDIES ON HUMANS: Several experimental investigations concerning tetracycline phototoxicity using human volunteers, not taking tetracyclines for therapeutic reasons, have been performed. The first two were initiated by several clinical reports about photoreactions soon after the introduction of DMCT. Harber et al studied test persons given DMCT 0.6 g, tetracycline 1.0 g daily and placebo combined with natural sunlight or filtered carbon arc lamp irradiation in a double blind test procedure assessing erythema

intensity, erythema threshold and erythema persistence (57). Their results indicated a possibly increased reactivity with DMCT combined with natural sunlight; however reactions were weak and inconsistent. With artificial irradiation no effects whatsoever were noted. Cahn et al studying 74 subjects on DMCT 0.6 g daily found 15 developing increased erythema when irradiated with 1 MED of carbon arc or Westinghouse Sun Lamp radiation, these reactions not occurring with windowglass filtering (19). In the same investigation 15 out of 64 subjects on DMCT and exposed to 1 MED of natural sunlight developed increased erythema, the reaction being prevented by windowglass filtration. Schorr and Monash investigating DMCT and tetracycline administered intradermally to 6 test persons and irradiated with 1 MED of natural sunlight (5 out of 6) or hot quartz lamp radiation (1 out of 6) demonstrated increased sensitivity with DMCT, and to a lesser degree with tetracycline, combined with natural sunlight with or without filter (58).

With the later derivatives, three comparative trials have been performed where volunteers have been given one of the two tetracycline derivatives or placebo in a double blind fashion and evaluated for abnormal objective as well as subjective reactions when exposed to full body natural sunlight. Thus Blank et al testing DMCT vs doxycycline (0.6 g and 0.2 g daily respectively) found 9/10 and 2/10 respectively with abnormal reactions (59). Frost et al comparing DMCT vs methacycline (0.6 g daily) noted 12/13 and 1/14 respectively with abnormal reactions (60). Frost et al investigating doxycycline vs minocycline (0.2 g daily) recorded 11/15 and 0/17 respectively with exaggerated reactions (61).

Rosén and Swanbeck tested 10 volunteers with doxycycline (0.1 g daily) combined with graded doses of UVA with a high intensity UVA lamp and found one with lowered UVA threshold dose (open study) (62).

PHOTOCHEMISTRY: Several photochemical methods have been applied in studies on the more basic events taking place during the initial steps of a phototoxic tetracycline reaction. Some of these will be briefly mentioned; Photochemical degradation: Irradiation of tetracycline solutions equilibrated with air changes the absorption spectra with a decrease of the major peak in the 365-375 nm region and the appearance of a new peak round 430 nm with chlortetracycline, DMCT and methacycline and a peak round 530 nm for the other derivatives (63, 64, 65).

Photooxidation: The oxygen uptake with different tetracycline derivatives in solution holding pH 7.0 irradiated with UVA showed chlortetracycline, doxycycline and DMCT to consume more oxygen than the other derivatives (minocycline with no uptake whatsoever) (64) which is fairly comparable to the order of rate constants for UVA-induced photodegradation (65). Studies on photooxidation of 2,5-dimethylfuran and dl-limonene showed both to be oxidized significantly (indicating the participation of singlet oxygen) in the presence of all tetracyclines except minocycline (64, 66).

Oxygen dependence: This has been shown with tetracycline induced photohemolysis (48), photodamage of human leucocytes (67) and photodamage of the thymidine incorporation into peripheral blood mononuclear cells as indicated by enhanced toxicity in a D₂O environment (65).

Cholesterol oxidation: Isolation of cholesterol-5 α -hydroperoxide from a photooxidized membrane is a method by which the involvement of singlet oxygen in the primary oxidation can be conclusively demonstrated (44). Irradiation of cholesterol in the presence of any of the tetracycline derivatives has shown this lipid peroxide to be produced, indicating singlet oxygen production (68).

DNA alterations: Two types of DNA injury can be produced by the tetracycline derivatives combined with UVA; (i) alteration of guanine residues leading to termination of DNA replication; (ii) breakage of the sugar-phosphate backbone (68). Alterations of guanine residues involve production of singlet oxygen since sodium azide strongly reduced the reaction. Breakage of the sugar-phosphate backbone was shown to be initiated by hydroxyl radicals as shown by electron paramagnetic resonance (EPR) spin trapping experiments.

ACTION SPECTRUM

HUMANS: As pointed out earlier the initial reports concerning the active wavelengths producing tetracycline photoreactions were conflicting. Most investigators found the action spectrum to be in the UVB since window-glass filtration inhibited the reaction in provocation experiments (19, 20, 31, 57) while clinical observations indicated that the reaction could be elicited through windowglass (27). The necessity of adding UVB radiation might have been due to insufficient doses of UVA given. Later expe-

riments with intradermal injection (5, 58) or oral administration of tetracyclines (23, 62) combined with natural sunlight or effective artificial UVA sources have shown the action spectrum to be in the UVA.

MICE: Monochromator studies on mice injected with DMCT showed an action spectrum extending from 350 to 420 nm with a peak at 400 nm i.e. in the UVA region (50). Measurements of phototoxic mouse tail edema with intraperitoneal injection of tetracycline showed positive reactions with UVA but negative ones with UVB (52).

RBCT: In photohemolysis studies with DMCT and irradiation with a high pressure xenon arc lamp insertion of a windowglass filter did not prevent cell lysis (47).

RELATIVE PHOTOTOXIC POTENCY

HUMANS: Referring to previously mentioned clinical reports concerning larger patient materials which permitted frequency calculations and scattered clinical reports the order of phototoxic potential seems to be: DMCT > chlortetracycline > doxycycline > tetracycline, oxytetracycline, lymecycline > methacycline, minocycline (for references see table 1). Comparative experimental studies on humans indicate the following order of potency DMCT > doxycycline > tetracycline, methacycline > minocycline (57, 58, 59, 60, 61) (see page 21).

MICE: Erythema readings on hairless mice revealed DMCT to be a more potent photosensitizer than tetracycline (51). Quantitative registration of phototoxic mouse tail edema showed the following order of photosensitizing capacity: doxycycline > DMCT > lymecycline > chlortetracycline, oxytetracycline, tetracycline, methacycline, minocycline (all negative) (52).

LYMPHOCYTES and LEUCOCYTES: Inhibitory effect on $[^3\text{H}]$ thymidine incorporation into lymphocytes by tetracycline photosensitization was in order: doxycycline > DMCT, chlortetracycline > methacycline > tetracycline, oxytetracycline > minocycline (inactive) (65). Inhibitory effects on oxygen consumption of phagocytosing leucocytes revealed: doxycycline > DMCT > oxytetracycline, lymecycline, chlortetracycline > minocycline (inactive)

(67).

PHOTODEGRADATION and PHOTOOXIDATION: Photodegradation of irradiated tetracyclines in solution (65) and oxygen uptake during irradiation (64) showed the following ranking: chlortetracycline, DMCT, doxycycline > tetracycline, oxytetracycline, methacycline > minocycline (inactive). Photooxidation of limonene and 2,5-dimethylfuran ranked chlortetracycline, DMCT, tetracycline (sic!) > methacycline, doxycycline, oxytetracycline > minocycline (64). In studies on transformation of cholesterol to its 5 α -hydroperoxide derivate by tetracycline photosensitization the efficiency seemed to be similar for all sensitizers except tetracycline which appeared to be more effective than the others (68).

DNA ALTERATIONS: Alterations of guanine residues leading to termination of DNA replication could be shown with all derivatives including minocycline while DNA breakage was most pronounced with doxycycline, less with DMCT, chlortetracycline and tetracycline, still less with oxytetracycline and not existent with minocycline (68).

MODE OF ACTION

As is evident from previously mentioned experimental studies, tetracycline photosensitization may primarily engage many types of biological molecules such as membrane lipids and DNA. Concerning DNA lesions, these have been shown to have their origin in the generation of two reactive oxygen species, namely singlet oxygen and hydroxyl radicals (68). This dependence on oxygen has also been shown in photohemolysis (48) and photosensitized damage to leucocytes (67) and lymphocytes (65).

Hasan et al postulate that aside from the mentioned photosensitization by the drug of the biological molecule to cause phototoxicity, the drug may also absorb light to yield one or more photoproducts absorbing visible light causing additional photoreactions (65).

Additional factors such as the relative binding of different tetracyclines to cellular components and serum proteins as well as relative lipophilicity may influence their photosensitizing ability in vivo. For example it has been shown that the different derivatives vary in lipophilicity and binding to human serum proteins in the following order: doxy-

cycline > methacycline, DMCT > tetracycline > oxytetracycline (69). Data concerning the subcellular sites of tetracycline photosensitized damage are not available. Such data are of importance since it has been shown that different photosensitizers have an affinity for certain cellular components leading to specific injury to these sites. Thus Allison et al showed anthracene and uroporphyrin I to be concentrated in the lysosomes destroying these when irradiated while rose bengal remained at the cell surface leading to cell membrane damage when irradiated (70). Fritsch et al demonstrated that haematoporphyrin is able to act by both mechanisms, depending on the length of incubation (71).

3. PRESENT INVESTIGATION

A. AIMS

The intention of the present investigation was to rank the phototoxic potency of the different tetracycline derivatives available on the Swedish market, supplemented with minocycline (see fig 3), by the use of several experimental in vitro as well as in vivo methods (including human volunteers). The detailed aims of each investigation were;

- I. To accept or reject the photohemolysis model as being suitable for studying DMCT phototoxicity, to investigate the relative potency of DMCT compared to chlorpromazine (CPZ) phototoxicity and to study the photohemolytic properties of separate parts of the UV spectrum without a photosensitizer.
- II. To compare the relative photohemolytic potency of all the tetracycline derivatives and relate this to clinical reports on phototoxicity.
- III. To compare the relative phototoxic potency in a system consisting of human fibroblasts in culture, being dividing nucleated cells with cell organelles as opposed to RBC.
- IV. To study the relative phototoxic effect of doxycycline and lymecycline (the two most commonly prescribed tetracyclines on the Swedish market) in human volunteers in a controlled study including DMCT.
- V. To study, in view of the experienced difficulty to provoke tetracycline phototoxicity in humans using artificial UVA, a possible augmentative effect exerted by UVB on phototoxicity elicited by doxycycline and UVA using the quantitative mouse tail technique.
- VI. To study in retrospect the number of patients with tetracycline phototoxicity consulting dermatology departments in Sweden during 1983, -84 and -85 and to relate this to prescribed quantities of the separate derivatives.

B. MATERIALS AND METHODS

DRUGS. The 8 commercial tetracycline derivatives (fig 3) were obtained from the following companies: DMCT, tetracycline, chlortetracycline and minocycline from Lederle Laboratories, Pearl River, New York; doxycycline,

oxytetracycline and methacycline from Pfizer, Brussels, Belgium; and lymecycline by Farmitalia Carlo Erba, Täby, Sweden. Chlorpromazine was obtained from AB Leo, Helsingborg, Sweden.

PHOTOHEMOLYSIS (I-II). A modification of the photohemolysis technique described by Kahn and Fleischaker was used (43), see fig 4.

RBC were obtained by venipuncture from healthy human volunteers not taking any drugs. RBC were drawn into ACD and then immediately washed in physiologic saline and centrifuged for 10 min at 2000 rpm. This procedure was repeated 3 times, whereafter the RBC were stored refrigerated no longer than 40 h before use to avoid spontaneous hemolysis.

The test drug was dissolved in a buffer solution consisting of 0.075 M Na-Veronal (barbital) at pH 7.4 with saline to 290 mosmol/kg. Ten milliliters of the buffered drug solution was poured into plastic cups forming a liquid layer of 7 mm. RBC 20 microliters were added to each cup, giving a dilution of 1:500. The cups were then irradiated with UVA, UVB or UVC. A metal halogen lamp (Osram-Ultramed 400 W) with an emission peak at 365 nm was used as the UVA source. A UVB filter consisting of 3-mm windowglass was inserted between the lamp and the samples, giving an output in UVA of $10 \text{ mW/cm}^2/\text{s}$ at 30 cm distance as measured with a PUVA-meter (Waldmann AG, Schwenningen, G.F.R.) For UVB 2 fluorescent tubes (FS 40 Westinghouse Sun Lamp, 40 W) emitting continuously from 280-380 nm with a peak at 313 nm were used, giving an output of $2.3 \text{ mW/cm}^2/\text{s}$ in the UVB region as measured with a UV-meter (Waldmann AG). For UVC one fluorescent tube (Philips TUV 30 W) emitting at 254 nm was used, having an output of $2.1 \text{ mW/cm}^2/\text{s}$ as measured with a UVX digital radiometer (Ultra-violet products Inc, San Gabriel, Calif., USA).

Duplicate samples of drug and RBC, RBC only, as well as one sample containing drug only (the last allowing compensation for possible UV-induced colour changes of the drug) were irradiated and controls kept in the dark. After irradiation the samples were incubated in the dark. The volume of the samples was then measured to compensate for minor losses due to evaporation and suspensions were transferred to test tubes and centrifuged for 10 min at 2000 rpm. Four milliliters of the supernatant was mixed with 1 ml of Drabkins' solution $\left[\text{K}_3\text{Fe}(\text{CN})_6 \text{ 200 mg, KNC 50 mg, KH}_2\text{PO}_4 \text{ 140 mg, surfactant Triton X-100 0.5 ml, diluted with distilled water to 1000 ml with pH 7.0-7.4} \right]$ in order to convert all types

of hemoglobin to methemoglobin which forms the stable pigment cyanmethemoglobin with a maximum absorption at 540 nm (72). This procedure further dilutes the hemoglobin to 1:625. The samples were read at 540 nm in a Beckman DB-GT spectrophotometer. A sample with 100% hemolysis consisted of 20 microliters of RBC in 12.5 ml of Drabkin's solution, also giving a dilution of 1:625.

All results are expressed as % hemolysis relative to the 100% hemolyzed solution using the following formula:

$$\frac{(EDR - ED)V - D}{T} \times 100$$

- where EDR = optical density of exposed drug solution with RBC
ED = optical density of exposed drug solution without RBC
D = optical density of dark control drug solution
T = optical density of total hemolysis control solution
V = $\frac{\text{volume after irradiation}}{\text{volume before irradiation}}$

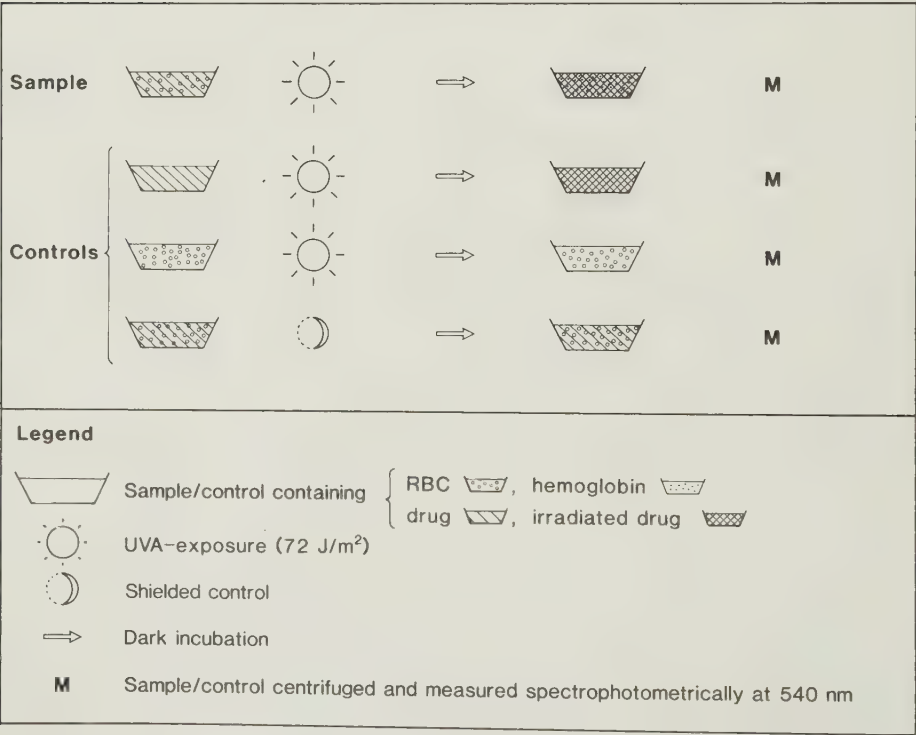


Fig 4. Schematic illustration of the photohemolysis technique.

FIBROBLAST CULTURE (III). A modification of a technique, earlier described for culture of human skin fibroblasts in order to study connective tissue components and lysosomal enzymes, was used (73).

Skin biopsies from healthy males were cut into small pieces, and placed at the bottom of 25 cm² plastic culture flasks (A/S Nunc, Denmark). The flasks were turned upside down and 5 ml medium was added. The biopsies were left uncovered with medium for 1-1.5 h to ensure adhesion before the flasks were turned again to be left untouched during 10 days at 37°C for outgrowth. All medium components were obtained from Gibco, Chemoferm AB, Sweden. Minimum essential medium with Hanks' salts was supplemented with heatinactivated fetal calf serum (10%), L-glutamine (585 mg/liter), nonessential amino acid mixture (1%) and gentamicin (100 mg/liter). During outgrowth the medium was changed twice a week. Confluent cells were subcultivated by the use of 0.05% trypsin - 0.002% EDTA.

For the experiments confluent cells from passage 1-6 were used. During irradiation of the flasks the medium was replaced by the drug dissolved in physiologic saline adjusted to pH 7.2 with NaOH. Nonirradiated drug-exposed cell cultures were kept in the dark for equally long periods as controls. Controls that were neither irradiated nor treated with drug were kept in the dark in physiologic saline.

The cells were irradiated in the flasks at 10 cm distance with UVA from 2 blacklight fluorescent tubes (Philips TLA 40 W/08) with an emission peak at 360 nm. A 3-mm window glass filter was inserted to eliminate wavelengths shorter than 320 nm. The output inside the flask at the level of the cells was 2.1 mW/cm²/s in the UVA as measured with a PUVA-meter.

After irradiation the drug solution was poured off and the cells in each flask were trypsinized, counted in a Bürker chamber and subcultivated into a tissue culture multiwell plate containing 12 wells with an area of 4.5 cm² (Linbro, Linbro Scientific Inc., Hamden, Connecticut, USA). The initial cell density of a control well was approximately 1.0×10^5 cells per well. The cells were then left to grow and were counted on days 1, 3, 7, 10, 14 and sometimes 21 with 2 wells counted on each occasion.

All experiments comparing different drugs were performed simultaneously with cells from one donor.

A schematic illustration of this technique is showed in fig 5.

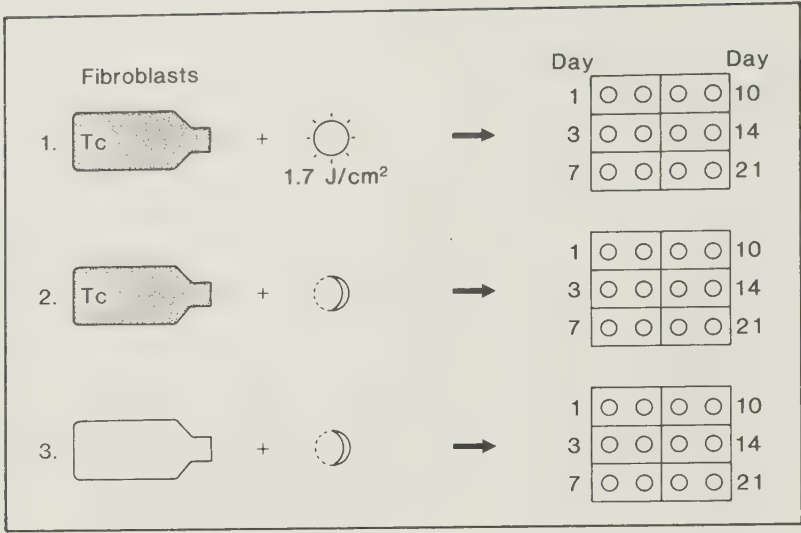


Fig 5. Illustration of the technique for phototoxicity studies with tetracyclines (TC) using fibroblast cultures. Sample 1 testing TC phototoxicity, sample 2 testing TC toxicity, sample 3 control cells. After exposure the cells are subcultivated and counted on several occasions.

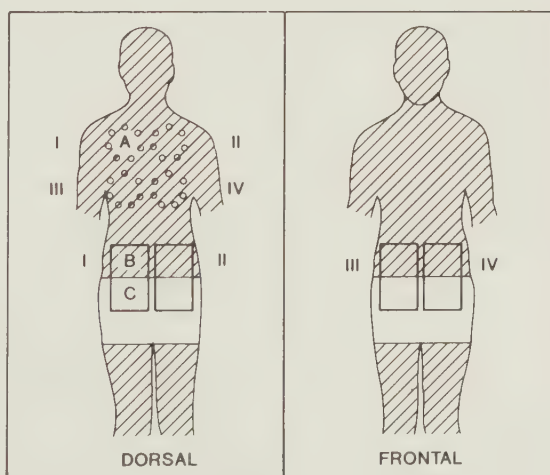
HUMAN VOLUNTEERS (IV). A double blind cross-over study on phototoxicity to three tetracycline derivatives was performed in 8 human volunteers. The three derivatives chosen were doxycycline and lymecycline, the two most commonly prescribed tetracyclines on the market (74% and 14% respectively of total daily doses prescribed in Sweden 1985), and for comparison DMCT, the tetracycline most often reported to be phototoxic in the literature though very little used in Sweden (0.003% of daily doses prescribed). Doxycycline and lymecycline were given in maximum recommended doses (twice the standard dose) and DMCT in the standard dose. In analysing phototoxic reactions with different modalities of artificial UVA, objective as well as subjective symptoms were considered, since in pilot experiments it was not possible to detect phototoxicity in 10 volunteers using artificial high intensity UVA and erythema readings only (unpublished observations).

Eight healthy volunteers (21-44 years), not on any systemic medication

for at least 5 days before the start of the study, were included. All medication was given for three days; DMCT 0.3 g twice daily, doxycycline 0.1 g twice daily and lymecycline 0.6 g twice daily. Placebo drug was given 2 tablets twice daily. All four preparations were tested randomly in a double blind cross-over fashion in each individual with a wash-out period of minimum 4 days.

Phototesting was done two hours after the last drug dose on day 3. For each drug two modalities of UVA were tested according to fig 6; (i) on the back 7 areas, measuring 3 cm^2 each, were irradiated with increasing doses of UVA (1.2 - 20.3 J) with an Osram Ultramed^R 400 W metal halogen lamp mounted in a Blue-Light-Spot^R housing equipped with filter h 1 (dr Hönle GmbH, Munich, G.F.R.) emitting continuously from 320 nm with a peak at 370 nm. The output at the level of the skin was $26 \text{ mW/cm}^2/\text{sec}$ in the UVA range as measured with a UVX digital radiometer (Ultraviolet Products Inc, San Gabriel, Calif., USA); (ii) an area measuring $12 \times 20 \text{ cm}$ of previously sunexposed skin on the lower back or abdomen was irradiated with 20 J of UVA with a PUVA 4000 - equipment (Waldmann AG, Schwenningen, GFR) emitting mainly in the UVA region with a peak at 365 nm; (iii) an area measuring $10 \times 12 \text{ cm}$ of previously unexposed skin on the lower back or abdomen was also irradiated with 20 J of UVA in the PUVA 4000-equipment. The tests were read blindly 24 hours later and the test persons were questioned about subjective side effects, mainly the sensation of stinging.

Fig 6. Experimental design showing two different phototesting modalities; A graded doses of UVA (1.2 - 20.3 J), B single dose of UVA 20 J given to sunexposed skin, C single dose of UVA 20 J given to unexposed skin. Phototesting was performed on 4 different occasions (I-IV) in combination with either DMCT, doxycycline, lymecycline or placebo.



Immediately before phototesting a 5 ml blood sample was drawn, the serum separated and kept at -22°C until analysed for tetracycline with a spectrophotofluorometric method using tetracycline as standard when lymecycline was measured (74).

QUANTITATIVE MOUSE TAIL TECHNIQUE (V). In view of the relative difficulty in provoking phototoxic erythema in volunteers with oral administration of tetracycline and artificial UVA (IV), it was decided to study the influence of additional doses of UVB on the phototoxic reaction elicited by doxycycline and UVA, this being the case when natural sunlight exposure is involved. The quantitative mouse tail technique, measuring phototoxic edema, was found most suitable for these experiments (75, 76).

Female albino mice were injected intraperitoneally with doxycycline 100 mg/kg bodyweight immediately before UVA irradiation. During exposure to UVR the animals were fixed in horizontal plastic tubes allowing only the tails to be exposed. For UVA 2 blacklight fluorescent tubes (Philips TLA 40 W/08) with an emission peak at 360 nm were used. A window glass filter was inserted to eliminate wavelengths shorter than 320 nm, giving an output at the level of the tails of $2.5 \text{ mW/cm}^2/\text{sec}$ in the UVA region as measured with a PUVA-meter. For UVB 2 fluorescent tubes (FS 40 Westinghouse Sun Lamp, 40W) were used, giving an output of $1.8 \text{ mW/cm}^2/\text{sec}$ in the UVB region as measured with a UV-meter.

The effect of relatively small (in the mouse) doses of UVB given in connection with the phototoxic treatment (doxycycline + UVA) was studied in 6 experiments. According to fig 7 UVB was given immediately before the phototoxic treatment in experiments no. 1 and 2, immediately after in experiment no. 3 and 24 hours before in experiments no. 4, 5 and 6.

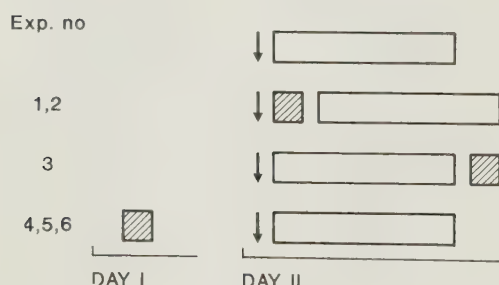


Fig 7. UVB given at different time intervals in connection with the phototoxic treatment (doxycycline + UVA) in the 6 experiments. UVA: open bars. UVB: filled bars. Doxycycline injection: ↓. Standard procedure i.e. without UVB is shown at the top.

To determine whether the addition of UVB had an additive or augmentative effect 5 groups of 10 animals were needed in each experiment (group I-V). Thus group I was given UVA only, serving as a control since previous and fresh pilot experiments had shown that this treatment induces no inflammatory reaction whatsoever. Group II was given UVB only, group III UVB and UVA, group IV doxycycline and UVA and group V UVB, doxycycline and UVA.

The animals were sacrificed 24 h after the beginning of UVA exposure. A piece of the tail was excised, weighed, dried at 110°C and weighed again. Results are presented as percent wet weight increase over controls. To determine if the inflammatory response in group V (each animal was treated with the full combination UVB, doxycycline and UVA) was greater than expected from simple addition, i.e. augmentation had taken place, this group was compared to an addition of the control group given only UVA (wet weight % in group I), the UVB edema (wet weight % in group II minus I) and the phototoxic edema (wet weight % in group IV minus I). The comparison of group V to the added values $[I + (IV-I) + (II-I)]$ was made using Student's t-test.

INCIDENCE STUDY. In an attempt to correlate the experimentally found ranking of phototoxic potency with tetracycline photoreactions in clinical reality different methods were considered. For information concerning epidemiologic data the Drug Information Committee, Swedish National Board of Health and Welfare was believed less suitable since reporting to this department can be suspected of being limited and selected. Instead all 39 dermatological departments in Sweden were invited to answer a questionnaire concerning all cases of tetracycline photoreactions that had attended during the years 1983, 1984 and 1985 according to registers on diagnosis. The incidence thus reported for each derivative was compared to calculated daily doses prescribed during the same years on the Swedish market.

C. RESULTS AND DISCUSSION

PHOTOHEMOLYSIS (I-II)

PHOTOHEMOLYTIC EFFECT OF UVR WITHOUT PHOTSENSITIZER (I). See fig 8. Only the largest dose of UVA (144 J/cm^2) induced a slight hemolysis. With UVB doses exceeding 4.1 J/cm^2 an increasing rate of hemolysis was found with almost total hemolysis with 16.6 J/cm^2 . With UVC total hemolysis was achieved with 3.8 J/cm^2 .

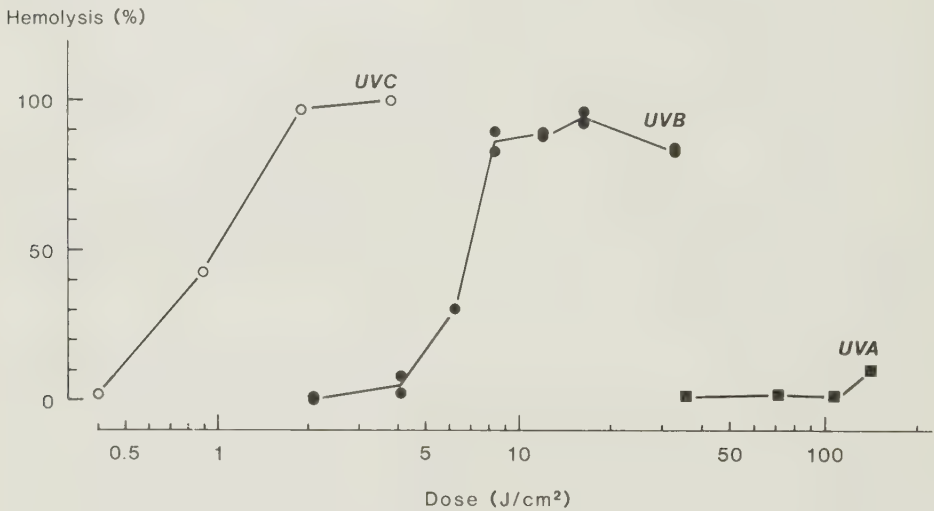


Fig 8. Dose-response curves for photohemolysis produced with UVC, UVB and UVA. Incubation time 2 h. The UVB curve represents the mean of two experiments.

Discussion: Thus substantial differences were found between the hemolytic capacity of different qualities of UVR with UVC as the most effective one, followed by UVB and to a very small degree by UVA causing hemolysis only with exceedingly large doses. The strong hemolytic properties of UVC have earlier been pointed out by Cook who found, using a low pressure mercury arc, that radiation of 185 nm was 100 to 200 times more effective than radiation of 254 nm (42). Later investigations of the wavelength range 240-300 nm have shown the action spectrum to descend from 240 nm with a minimum at 265 nm, a new peak at 280 nm, and then again descending

to an absolute minimum at 300 nm (77). The primary photochemical process may involve membrane protein in view of this action spectrum which resembles protein absorption (77). Another primary mechanism i.e. membrane lipid photoperoxidation has been proposed by Roshchupkin et al who found the hemolysis to be oxygen dependent and suggested a two step mechanism including (i) the inactivation of the membrane α -tocopherol and (ii) the photoperoxidation of the now unprotected neighboring phospholipids (78).

PHOTOHEMOLYTIC EFFECT OF DMCT AND CPZ (I): For RBC irradiated with 72 J/cm^2 of UVA in the presence of DMCT 100 microgram/ml a hemolysis rate of 90% was reached with 2 h of incubation which was chosen as standard incubation time in subsequent experiments.

With increasing doses of UVA and DMCT 25 microgram/ml a dose-response curve was obtained levelling off above 72 J/cm^2 motivating this as the standard exposure in later experiments.

With 72 J/cm^2 of UVA, 2 h incubation time and increasing concentrations of DMCT a dose-response curve was obtained with 88% hemolysis at 50 microgram/ml (fig 9).

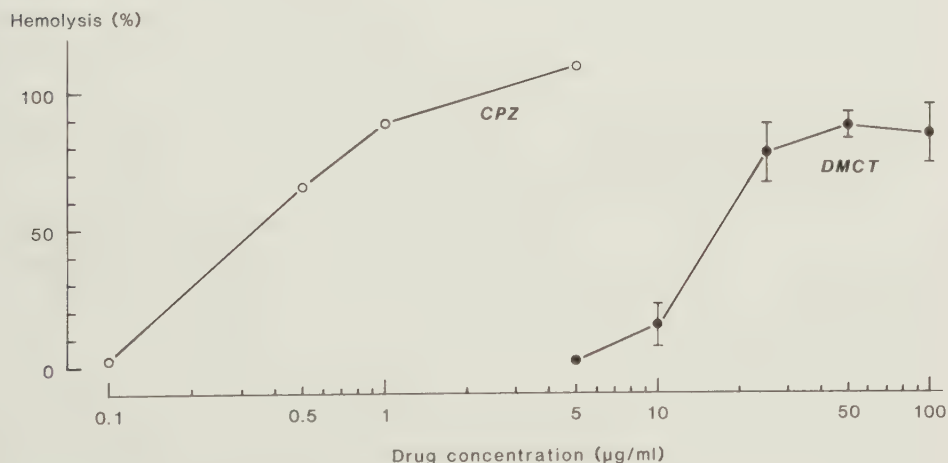


Fig 9. Dose-response curves for CPZ and DMCT photohemolysis with 72 J/cm^2 of UVA and incubation time 2 h. The values for DMCT are the mean of 10 experiments. Vertical bars indicate the standard deviation.

RBC irradiated with a subhemolytic dose of UVB (4.1 J/cm^2) in the presence of DMCT showed no hemolysis.

With 72 J/cm^2 of UVA, 2 h incubation time and increasing concentrations of CPZ a dose-response curve was obtained with 89% hemolysis at 1 microgram/ml (fig 9).

Discussion: DMCT in combination with UVA was thus shown to induce significant photohemolysis verifying this technique as suitable for studying DMCT phototoxicity. Earlier results in this field have been contradictory, some investigators reporting positive (47, 48, 49), other negative (43, 79) results. The previous negative results might have been due to insufficient doses of UVA, DMCT being a weak photosensitizer compared to, for example, CPZ, the latter causing hemolysis already at a concentration of 0.5 microgram/ml, a rate about 50 times that of DMCT (fig 9). Another photosensitizer, benoxaprofen which is a non steroidal antiinflammatory drug, has been shown to have intermediate hemolytic ability resulting in 74% hemolysis with a concentration of 50 microgram/ml and 11 J/cm^2 of UVA (corresponding data for DMCT being 88% hemolysis with 72 J/cm^2 of UVA) (80). The photohemolysis method was thus found superior to another phototoxicity screening method viz. the candida albicans inhibition test which has failed to detect DMCT phototoxicity (81).

It is important to compensate for possible colour changes of the irradiated drug solutions. UVA exposure of DMCT causes a pronounced colour change with the photoproducts absorbing at 540 nm which results in falsely high estimated hemolysis if not compensated for. This phenomenon was also observed by Kahn and Fleischaker (43).

The negative results in the UVB experiments are in accordance with the action spectrum of DMCT being in the UVA, as reported in recent investigations (47, 50) while early clinical reports indicated UVB as the effective radiation (19, 20, 31, 57).

HEMOGLOBIN ANALYSIS USING DRABKIN'S SOLUTION (I): RBC were irradiated with UVB $2.1 - 24.8 \text{ J/cm}^2$ without drug. For each UVB dose four cups were used, two of which were treated with Drabkin's solution (as was the 100% control) according to the basic experimental design, the other two with 1 ml of buffer instead. For the latter two samples the 100% control was hemolysed with distilled water and not treated with Drabkin's solution.

This experiment was repeated with RBC irradiated with UVA in the presence of DMCT.

When RBC without drug were thus irradiated with 8.3 J/cm^2 of UVB or more the analysis using Drabkin's solution gave higher values than the falsely low figure obtained with buffer alone, the largest discrepancy being 53 percental units with 24.8 J/cm^2 . The same tendency, though less pronounced, occurred with UVA and DMCT.

Discussion: Hemoglobin exists mainly in two forms, namely in its unoxygenated form symbolized as Hb and in its oxygenated form symbolized as HbO_2 . Hemoglobin and oxyhemoglobin have distinct absorption spectra; hemoglobin with a single band at 552.5 nm and oxyhemoglobin with two bands at 541.5 and 576 nm (82). Two methods for hemoglobin determination exist namely the oxyhemoglobin method and the cyanmethemoglobin method (83). With the oxyhemoglobin method blood is oxygenated by exposure to atmospheric oxygen and read at 540 nm, one disadvantage being the inability to include methemoglobin which under natural conditions only constitutes 1% of RBC hemoglobin. With the cyanmethemoglobin using Drabkin's solution practically all forms of hemoglobin are converted to cyanmethemoglobin absorbing at 540 nm. Since UVR is capable of converting oxyhemoglobin to methemoglobin (44), having its absorption peak at 630 nm, the oxyhemoglobin method is not suitable when UVR is involved. This problem was realized by Kahn and Fleischaker who named it the fading of hemoglobin, however these investigators did not propose an alternative method of analysis (43). However, this problem can be overcome by the use of the cyanmethemoglobin method as proposed to us by Johnson (personal communication 1981) and later Hetherington and Johnson (84).

PHOTOHEMOLYTIC POTENCY OF TETRACYCLINE DERIVATIVES (II): RBC in the presence of DMCT and doxycycline 5, 10, 25, 50 and 100 microgram/ml and methacycline, tetracycline, oxytetracycline, chlortetracycline, lymecycline and minocycline 10, 25, 50, 100 and 200 microgram/ml were irradiated with 72 J/cm^2 of UVA and incubated for 2 h.

As is evident from fig 10 DMCT and doxycycline showed pronounced hemolytic properties with a maximum of 88% and 85% hemolysis, respectively, at a concentration of 50 microgram/ml. A dose-response curve levelling off above 50 microgram/ml was obtained. Methacycline showed an intermed-

iate hemolytic effect of 36% at 200 microgram/ml. Tetracycline, oxytetracycline, chlortetracycline and lymecycline were weak photosensitizers while minocycline showed no hemolytic effect whatsoever.

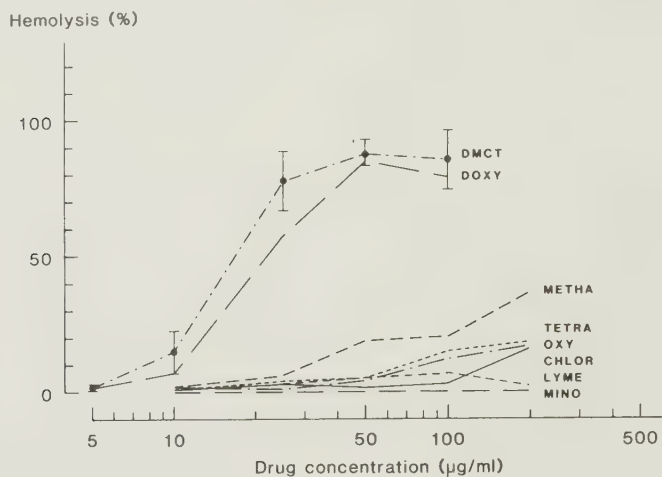


Fig 10. Dose-response curves for photohemolysis with the 8 tetracycline derivatives. RBC in increasing concentrations of drug were irradiated with 72 J/cm^2 of UVA and incubated for 2 h. The values for DMCT are the mean of 10 experiments, vertical bars indicating the SD.

RBC in the presence of DMCT, methacycline and oxytetracycline irradiated with doses of UVB previously shown to be subhemolytic caused no hemolysis.

RBC treated with UVA preirradiated DMCT and doxycycline induced no hemolysis.

Discussion: There thus seems to be a fairly good correlation between the ranking of photohemolytic potency of the different derivatives and the findings in several above-mentioned reports concerning relative phototoxic potential in humans undergoing tetracycline therapy (for references see table 1) experimental studies in humans (57, 58, 59, 60, 61), studies in mice (52), studies on lymphocytes (65) and leucocytes (67), experiments with photodegradation and photooxidation (65, 64) and the ability to cause DNA breakage (68). The only derivative deviating to some extent in

this respect was chlortetracycline which belongs to the group of less effective photohemolyzers but with a high ranking in other types of investigations.

DMCT, methacycline and oxytetracycline irradiated with UVB did not induce photohemolysis, confirming the action spectrum to be in the UVA.

DMCT and doxycycline preirradiated with UVA showed no hemolytic effect, indicating no formation of toxic photoproducts, which is the case in CPZ photohemolysis as reported by Johnson (45, 46).

FIBROBLAST GROWTH INHIBITION (III).

STUDIES ON DOXYCYCLINE AND CPZ: UVA doses of 2.5 J/cm^2 or less did not affect cell growth.

With 1.9 J/cm^2 of UVA and doxycycline 5, 10, 25 and 50 microgram/ml a dose-response relationship concerning growth inhibition was obtained with increasing concentrations resulting in total cell death with 25 and 50 microgram/ml (fig 11).

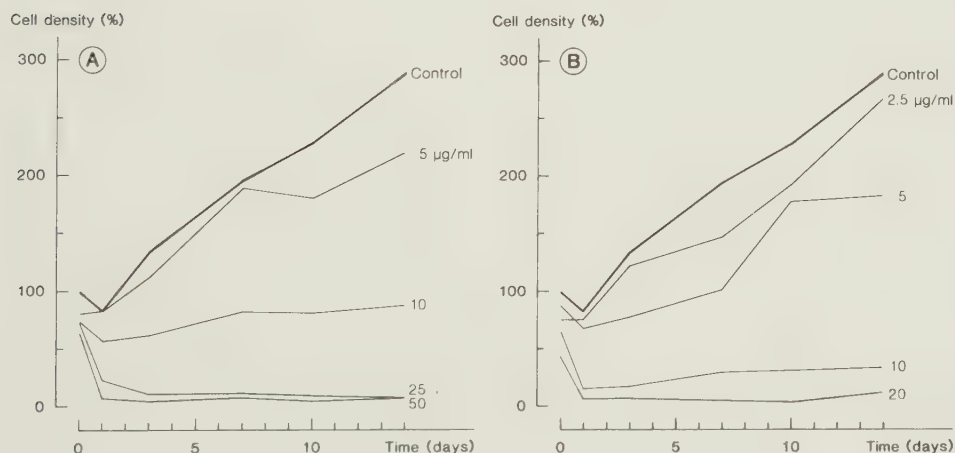


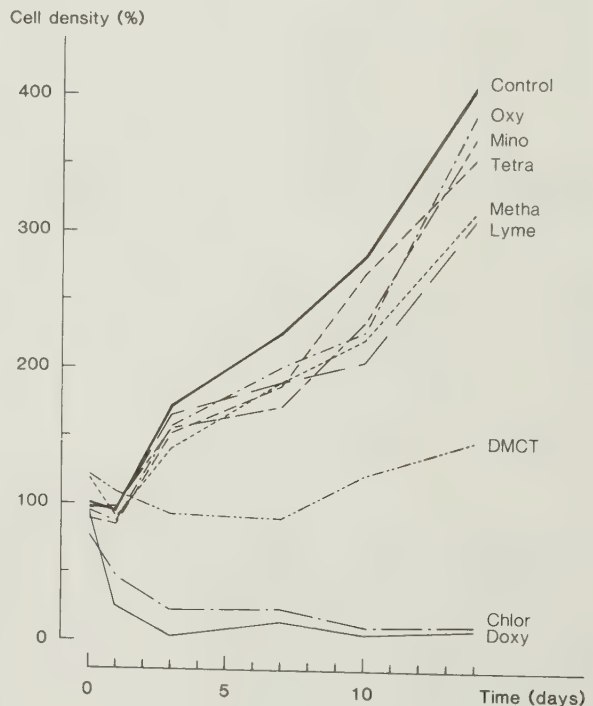
Fig 11. Fibroblasts treated with different concentrations of doxycycline (A) and CPZ (B) and 1.9 J/cm^2 of UVA on day 0 compared to untreated cells. Cell density of control cells immediately after irradiation on day 0 is plotted as 100% and serves as base line control for all curves.

A dose-response curve was also obtained with a constant doxycycline concentration (50 microgram/ml) but increasing doses of UVA with a total cell death at 1.9 J/cm^2 .

With CPZ 2.5, 5, 10 and 20 microgram/ml combined with 1.9 J/cm^2 of UVA total cell death took place with 20 microgram/ml (fig 11).

STUDIES ON TETRACYCLINE DERIVATIVES: Fibroblasts in the presence of 50 microgram/ml of each of the 8 different tetracycline derivatives were irradiated with 1.9 J/cm^2 of UVA. As can be seen from fig 12 chlortetracycline and doxycycline addition both resulted in a cell number decreasing to almost zero on day 3 with no tendency to recovery during 14 days' observation. DMCT supplementation resulted in a temporary arrest in cell growth, during 7 days, after which cell growth recurred. The other tetracyclines tested had only weak or no phototoxic influence on cell growth at the concentration tested.

Fig 12. Cell growth pattern for fibroblasts treated with oxytetracycline, minocycline, tetracycline, methacycline, lymecycline, DMCT, chlortetracycline and doxycycline 50 microgram/ml and UVA 1.9 J/cm^2 on day 0 compared to untreated cells.



Discussion: Doxycycline and chlortetracycline followed by DMCT were the strongest photosensitizers in this investigation in fairly close correla-

tion with the results obtained using the photohemolysis technique. The additional finding of chlortetracycline as a strong photosensitizer further confirms the close correlation with other clinical and experimental reports as discussed above (page 38).

Investigations on dividing fibroblasts may constitute a more physiologic system than the use of erythrocytes, which are devoid of nuclei and without the capacity to divide. This is also indicated by the lower and more physiologic doses of drug and UVA applied, affecting cell growth with doxycycline concentrations as low as 5-10 microgram/ml which is in the same order of magnitude as normal serum concentrations in humans (2-4 microgram/ml) (85).

CPZ was shown to be a more potent photosensitizer than doxycycline in accordance with the photohemolysis experiments. The ability of CPZ to induce growth inhibition in human fibroblasts verifies the findings of Ljunggren et al who in addition found DNA-interactions as one of the possible underlying phototoxic mechanisms (86).

PHOTOTOXICITY IN HUMANS (IV)

GRADED DOSES OF UVA TO SMALL AREAS OF SKIN: Erythematous scores with graded UVA doses ($1.2-20.3 \text{ J/cm}^2$) were added in each subject. Reactions stronger than with placebo developed in 3/8 persons taking DMCT, 6/8 taking doxycycline and 5/8 taking lymecycline. Comparing different drugs, however, must be done with caution since the reactions were weak and did not in all cases exceed that of placebo. When only strong reactions were included, one reaction on each of the three derivatives was recorded.

SINGLE DOSE OF UVA TO LARGE AREAS OF SKIN: In 5 of the subjects taking placebo UVA produced erythema in itself, making weak reactions with tetracyclines of no significance. However, two strong reactions were encountered, both with doxycycline and in "non-exposed" skin.

SUBJECTIVE REACTIONS: When graded doses of UVA were administered 2/8 persons on DMCT, 4/8 on doxycycline and 2/8 on lymecycline experienced a sensation of stinging exceeding that with placebo and UVA. With a single dose of UVA to large area of skin, stinging was never recorded with placebo, DMCT or lymecycline as opposed to doxycycline which produced

this effect in 3/8 persons.

ADDITION OF ALL REACTIONS: In order to estimate the relative phototoxic potential all types of substantial reactions with the two irradiation modalities were added for each individual according to table 2. Using this method of ranking reactions of any kind were recorded in 7/8 provocations with doxycycline and in 2/8 with DMCT and lymecycline respectively.

			Individual no.							
			1.	2.	3.	4.	5.	6.	7.	8.
DMCT	Serum conc.		4.0	1.1	2.5	3.2	1.9	4.2	8.1	3.6
	Graded UVA	Obj. Subj.			+				+	
	Single UVA	Obj. Subj.								
Doxy	Serum conc.		3.2	4.2	5.7	3.8	0.9	2.1	0.9	1.6
	Graded UVA	Obj. Subj.	+		+		+	+	+	
	Single UVA	Obj. Subj.		+	+			+		+
Lyme	Serum conc.		8.5	3.5	4.4	6.0	1.1	2.3	2.0	2.0
	Graded UVA	Obj. Subj.	+						+	
	Single UVA	Obj. Subj.								

Table 2. Added objective and subjective reactions judged clinically relevant with graded and single doses of UVA. Serum levels of drug are included (microgram/ml).

SERUM LEVELS: The following tetracycline levels were recorded (standard deviation in brackets): DMCT 3.6 ($\pm$ 2.1) microgram/ml, doxycycline 2.8 ($\pm$ 1.7) microgram/ml and lymecycline (measured as tetracycline) 3.7 ($\pm$ 2.5) microgram/ml.

Discussion: The present data indicate that doxycycline is a more potent photosensitizer than lymecycline. Concerning the comparatively few reactions with DMCT, it should be noted that this drug was given in the

recommended normal dose only, while the two others were given in double the recommended normal dose. The findings concerning doxycycline and lymecycline are in accordance with the ranking obtained in erythrocytes (II), fibroblasts (III) and several other in vitro and in vivo experimental studies as discussed previously. They are also consistent with the few clinical reports on lymecycline phototoxicity (32) compared to doxycycline phototoxicity (29, 32, 35, 36, 37, 38). The relatively strong photosensitizing capacity of doxycycline has also been shown in a comparative study versus minocycline in humans (61).

Phototoxicity to tetracyclines administered by mouth is difficult to produce in comparison with for example 8-methoxypsoralen (8-MOP) (87). In particular this seems to be the case when artificial UVA or windowglass filtered natural sunlight is employed (19, 20, 23, 31, 57, 62 Bjellerup and Ljunggren, unpublished results). Phototoxic reactions were more easily detected when subjective symptoms were also recorded.

Theoretically, it should be possible to provoke tetracycline photoreactions, being of the phototoxic type, in all individuals provided that the tetracycline concentration and UVA dose delivered to the target organ are sufficiently large. The fact that not every individual reacted and the absence of correlation between tetracycline serum levels and photoreactivity in this study (see table 2) indicates that individual factors are of importance. Different tetracycline derivatives produced different serum levels in one and the same individual and, as pointed out earlier, these levels did not correlate with photoreactivity. This fact however does not exclude a reciprocal relationship between serum levels, with different doses of one and the same derivative, and photoreactivity in a single individual. This is the case with 8-MOP which produced varying serum levels in different individuals and a poor relationship between serum concentration and photoreactivity comparing different individuals. However, within a single individual, there was a clear-cut reciprocal relationship between dose, serum level and photoreactivity (87).

UVB-AUGMENTATION (V)

The results of the 6 experiments are shown in fig 13.

UVB IMMEDIATELY BEFORE THE PHOTOTOXIC REACTION: In experiments 1 and 2

UVB 4.3 J/cm² and 2.2. J/cm² was given immediately before irradiating doxycycline treated animals with UVA 45 J/cm². In both experiments the wet weight increase was higher than expected from addition (difference 3.7 and 1.9 percental units respectively, the difference being statistically significant in experiment 1).

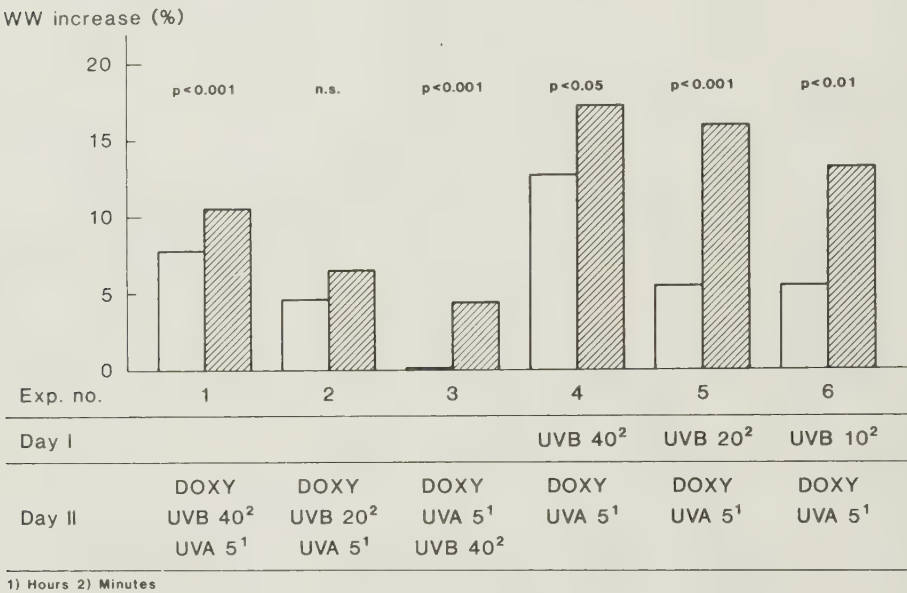


Fig 13. Results of UVB addition to the phototoxic reaction from doxycycline + UVA, testing the effect of varying UVB doses and different time intervals. For each of the 6 experiments a comparison between a calculated simple additive effect (open columns) and the *de facto* augmentative effect (filled columns) is made. The height of the columns shows the wet weight increase over base line values.

UVB IMMEDIATELY AFTER THE PHOTOTOXIC REACTION: In experiment 3 UVB 4.3 J/cm² was given immediately after UVA exposure of doxycycline treated animals. The wet weight increase was significantly higher than expected (difference 4.3 percental units).

UVB 24 h BEFORE THE PHOTOTOXIC REACTION: In experiments 4, 5 and 6 UVB 4.3 J/cm², 2.2 J/cm² and 1.1 J/cm² was given 24 h before doxycycline

and UVA resulting in wet weight increases significantly higher than expected from addition (difference 4.5, 10.3 and 7.5 percental units respectively).

Discussion: The addition of small doses of UVB to the phototoxic doxycycline reaction thus increased the inflammatory mouse tail edema more than could be explained by simple addition, i.e. photoaugmentation was demonstrated. When UVB was given in immediate connection with the phototoxic reaction the UVB/UVA order was of no importance (exp. no 1, 2 and 3).

Solar dermatitis, not in combination with photosensitizers, is mainly caused by UVB but recent data indicate that the cutaneous response is the result of an action by different wavelengths of UVR and visible radiation (88). Thus Willis et al found a reinforcing effect of preexposure with UVA on UVB erythema in man terming this phenomenon "photoaugmentation" (89). The phenomenon of UVA photoaugmentation of UVB erythema was confirmed by Kaidbey and Kligman who also could demonstrate UVB-photoaugmentation of the phototoxic erythema produced by external application of coal tar and 8-MOP in combination with UVA (90). The principle of photoaugmentation was denied by Ying et al who explained the co-effect of UVA and UVB without a photosensitizer by simple addition (91). Again, the experiments of Spiegel et al (92) speak in favour of an augmentative effect. However all the investigations cited relied on erythema readings in man and were thus not quantitative. A later study, using the quantitative mouse tail technique, was able to confirm the augmentative effect of UVB given immediately in connection with the phototoxic reaction elicited by UVA and internally administered CPZ, 8-MOP and chlordiazepoxide (93). In accordance with the present study the UVB/UVA order was of no importance.

In the present study the addition of UVB 24 h before the phototoxic treatment resulted in an even stronger augmentation, maximally causing a wet weight increase % five times higher than when the same UVB dose was given in immediate connection with UVA (fig 13 exp.no. 2 and 5). A possible explanation of this is the increased concentration of tetracycline in inflamed skin as has been reported in man (15, 34). In this situation the augmentation is thus proposed to be a combination of the general augmentative effect independent of UVB/UVA order, seen in the other experiments (no 1, 2, 3) and the concentration of drug in UVB-inflamed tissue. It is thus concluded that UVB, although unable to induce

tetracycline phototoxicity in itself, significantly modifies the phototoxic reaction from doxycycline and UVA in different ways, not only by addition but by augmentation. This phenomenon may be of importance in clinical tetracycline phototoxicity elicited by natural solar irradiation (containing a mixture of UVB and UVA), and affords a possible explanation of the difficulties in provoking tetracycline phototoxicity in humans using artificial light sources emitting UVA only or window glass filtered natural sunlight (19, 23, 57, 62, IV, Bjellerup and Ljunggren, unpublished results).

INCIDENCE STUDY

Of the 39 dermatological departments addressed 23, including all the major departments, answered the questionnaire. In all, only 16 tetracycline photoreactions were recorded during the period 1983-85 of which 12 were reactions to doxycycline, 3 to oxytetracycline and 1 to tetracycline. Using the number of reactions with doxycycline as a reference and assuming all derivatives to be equally potent sensitizers, the expected number of reactions for each derivative was calculated using statistics on daily doses prescribed on the Swedish market during 1983-85, with the following result:

	DD $\times 10^6$	Reactions reported	Reactions expected
Doxycycline	19.1	12	12
Lymecycline	4.1	-	2.6
Tetracycline	1.2	1	0.8
Methacycline	0.9	-	0.6
Oxytetracycline	0.8	3	0.5
Chlortetracycline	0.3	-	0.2
DMCT	0.008	-	0.01

Significant conclusions should not be drawn from this study for several reasons, such as; (i) it is a retrospective study; (ii) many of the reacting patients have probably had their medication prescribed by a dermatologist making it plausible that a selection of certain tetracycline

derivatives is at hand not reflecting the prescription pattern for other physicians; (iii) selected information concerning avoidance of solar exposure has probably been given, underestimating the phototoxic potency of well known sensitizers. However, the result illustrates that phototoxic tetracycline reactions are rather uncommon. It also verifies that such reactions do occur with doxycycline. In agreement with the comparative study on doxycycline and lymecycline in humans (IV) reactions to doxycycline are more common than to lymecycline. However this difference is not significant using a binominal test (with correction $p = 0.108$).

4. GENERAL DISCUSSION

In this series of experiments the phototoxic potency of different tetracycline derivatives was investigated using two different in vitro experimental methods; photosensitized lysis of human erythrocytes and growth inhibition of cultured human skin fibroblasts. The phototoxic ranking thus obtained was tested in human volunteers in a double blind cross-over study including doxycycline and lymecycline (the two derivatives most often prescribed on the Swedish market), this study also included DMCT. In view of the difficulty with which these reactions were produced using an artificial UVA source and small area erythema readings, the studies were extended to investigate the possible role of UVB-augmentation in doxycycline phototoxicity using an in vivo method measuring inflammatory mouse tail edema. In addition a retrospective incidence study was made using registered cases of tetracycline photoreactions reported from dermatologic departments in Sweden.

Photohemolysis. Lysis of RBC could be obtained with all tetracycline derivatives except minocycline provided high enough concentrations of drug and large enough doses of UVA were employed. The 8 derivatives tested were found to have significantly different phototoxic capacities with DMCT and doxycycline as the most effective hemolyzers followed by methacycline with intermediate activity; tetracycline, oxytetracycline, chlortetracycline and lymecycline with weak activity. Minocycline had no hemolytic effect whatsoever. The photohemolysis technique was thus found suitable for studies on tetracycline phototoxicity. Further the photohemolytic ability of different qualities of UVR without photosensitizer was studied confirming the pronounced hemolytic ability of UVC, the intermediate ability of UVB and the minimal effect of UVA. The importance of using the cyanmethemoglobin method for hemoglobin determination was verified.

Fibroblast growth inhibition. As opposed to the RBC, the fibroblast is a cell with cell organelles including a nucleus and with the capacity to divide. This type of cell could be photosensitized, as is evident from growth inhibition, with much lower and thus more physiological doses of doxycycline and UVA. When testing all derivatives for relative phototoxic potency, substantial differences were detected. Thus chlortetracycline

and doxycycline 50 microgram/ml in combination with 1.9 J/cm^2 of UVA resulted in total cell death while DMCT at the same doses resulted in a temporary arrest in cell growth. The other derivatives tested had only weak or no phototoxic influence on cell growth.

The correlation thus seems to be very good between the phototoxic ranking obtained by use of the photohemolysis and the fibroblast growth inhibition techniques and results obtained by other investigators including reports on photoreactions in humans undergoing tetracycline therapy, experimental studies on humans, studies in mice, experiments with lymphocytes and leucocytes, studies on photodegradation and photooxidation and the ability to induce DNA-breakage.

Phototoxicity in humans. By use of different modalities of irradiation and registration of both objective and subjective symptoms in volunteers, treated with oral administration of DMCT, doxycycline and lymecycline in combination with artificial UVA, phototoxic reactions could be detected with all three derivatives. Comparing doxycycline and lymecycline, which were administered in comparable doses, doxycycline was the stronger photosensitizer provoking reactions of any kind in 7/8 test persons (lymecycline in 2/8) confirming the experimental in vitro ranking. The correlation between tetracycline doses, serum levels and reactivity was poor, indicating individual factors of importance. Reciprocity studies within individuals were not performed.

UVB-augmentation. The study on UVB augmentation of the phototoxic reaction with doxycycline + UVA, using registration of mouse tail edema, demonstrated two types of augmentation; (i) a general augmentative effect of UVB given either immediately before or immediately after the phototoxic reaction; (ii) an enhanced augmentative effect of UVB given 24 h before the phototoxic reaction possibly reflecting the increased concentration of doxycycline in inflamed tissue. The absence of UVB augmentation offers a possible explanation for the relative difficulty of provoking tetracycline photoreactions in humans using artificial UVA sources and filtered natural sunlight in this and other studies.

Incidence study. The present incidence study illustrates the relative rareness of tetracycline photoreactions and verifies the ability of doxycycline to induce such reactions.

Mechanisms: It was not the aim of the present investigation to study tetracycline phototoxicity on the subcellular or molecular level. Several

such mechanisms have been proposed by other investigators. On the molecular level the ability of tetracyclines to induce photooxidation of cholesterol and lecithin, alterations of guanine residues leading to termination of DNA-replication, breakage of the DNA sugar-phosphate backbone and photooxidation of tryptophan (94) have been demonstrated, all these reactions being dependent on the presence of oxygen. These abilities open the possibility for phototoxic interactions at several subcellular levels such as the cell membrane, lysosome membranes and nuclear constituents. It is also unknown which type of cell is most important in the initial phase of phototoxic injury subsequently leading to the biological response of erythema and edema. The endothelial cell may be of importance as is the case in photosensitivity induced by erythropoetic protoporphyria (95). In fact we have detected an increased sensitivity to doxycycline and UVA in cultured human umbilical vein endothelial cells in comparison with human fetal fibroblasts (Bjellerup and Kjellström, unpublished observations). Lately the role of complement and polymorphonuclear leukocytes in photoreactions caused by erythropoetic protoporphyria and in DMCT-phototoxicity in guinea pigs has been demonstrated (96, 97).

5. SUMMARY

The phototoxic potency of different tetracycline derivatives was investigated experimentally in two in vitro systems; (i) photolysis of erythrocytes (photohemolysis); (ii) growth inhibition in cultured human fibroblasts. Pronounced differences in phototoxic ability were found with the following order of potency; DMCT = chlortetracycline = doxycycline >> methacycline > tetracycline = oxytetracycline = lymecycline > minocycline.

In a double blind cross-over study in human volunteers the same order of relative phototoxic potential could be verified comparing doxycycline and lymecycline, using different modalities of artificial UVA radiation and recording objective as well as subjective reactions. No correlation was found between administered dose, serum level and reactivity indicating the importance of other individual factors.

UVB augmentation of the phototoxic reaction elicited by doxycycline and UVA was verified by investigations in mice registering inflammatory tail edema. Two types of UVB augmentation were detected; a general augmentative effect of UVB given either immediately before or after the phototoxic reaction and an enhanced augmentative effect of UVB given 24 h before the phototoxic reaction, possibly reflecting an increased concentration of doxycycline in inflamed tissue. The phenomenon of UVB-augmentation offers a possible explanation of present and previous difficulties in provoking phototoxic tetracycline reactions in humans using artificial UVA or filtered sunlight.

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Studies on Photohemolysis with Special Reference to Demethylchlortetracycline

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Bjellerup M, Ljunggren B. Studies on photohemolysis with special reference to demethylchlortetracycline. *Acta Derm Venereol (Stockh)* 1984; 64: 378-383.

Hemolysis induced by ultraviolet radiation as well as demethylchlortetracycline (DMCT) phototoxicity have been investigated in a model using human red blood cells. Total hemolysis for UV-B and UV-C was obtained with 8.3 and 1.9 J/cm² respectively. DMCT was shown to have pronounced hemolytic properties causing 88% hemolysis at 50 µg/ml and 72 J/cm² of UV-A. No increased hemolysis rate was seen in combination with UV-B. Several factors influencing the results were studied such as incubation time, UV-A dose, drug concentration and different methods for hemoglobin detection. Photohemolysis is an accurate tool for demonstrating DMCT phototoxicity. *Key words: Phototoxicity; Tetracycline.* (Received November 10, 1983.)

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Previous studies on the phototoxic effect of drugs have mainly focused on the psoralens and the phenothiazines. The tetracyclines, on the other hand, have been less extensively studied in spite of several clinical reports of light-induced reactions in the skin and nails (1). In experimental studies on phototoxicity different models have been used including the candida albicans inhibition test, photohemolysis, growth inhibition of mammalian cells in culture and registration of phototoxic erythema and edema in animals and human volunteers (2). Tetracyclines have been tested with negative result using the candida albicans inhibition method (3). Investigations using living mammalian cells in culture showed phototoxic effects from demethylchlortetracycline (DMCT) but no such effects from tetracycline (4). Experiments using human lymphoid cells showed decreased incorporation of thymidine into nuclear DNA after addition of DMCT, which was the only tetracycline tested (5). Studies on mice demonstrated a phototoxic edema from DMCT, doxycycline and lymecycline whereas other tetracyclines were negative (6). Using the photohemolysis test, which is considered to reflect membrane damage, Kahn & Fleischaker were unable to show phototoxicity from DMCT or tetracycline (7). Swanbeck & Wennersten, on the other hand, found DMCT to have a photohemolytic effect (8). In a recent study Hasan et al. (10), testing DMCT and tetracycline, could show no photohemolysis.

In view of these conflicting results we considered it of interest to reinvestigate DMCT using a photohemolysis method. Different parameters influencing the results have been evaluated.

MATERIAL AND METHODS

A modification of the photohemolysis technique described by Kahn & Fleischaker was used (7).

Drugs

Demethylchlortetracycline HCl (Declomycine®) was provided by Lederle Laboratories, Pearl River, N.Y., USA. Chlorpromazine HCl (Hibernal®) was provided by AB Leo, Helsingborg, Sweden.

Basic experimental design

Red blood corpuscles (RBC) were obtained by venipuncture from healthy human volunteers not taking any drugs. RBC were then immediately washed in physiological saline and centrifuged for 10 min at 2000 rpm. This procedure was repeated three times, whereafter the RBC were stored no longer than 40 h before use to avoid spontaneous hemolysis.

The drug was dissolved in a buffer solution consisting of 0.075 M Na-Veronal (barbital) at pH 7.4 with saline to 290 mosmol/kg. Ten ml of the buffered drug solution was poured into plastic cups forming a liquid layer of approximately 7 mm. RBC 20 μ l were added to each cup giving a dilution of 1:500. The cups were then irradiated with long-wave (UV-A), medium-wave (UV-B) or short-wave (UV-C) ultraviolet radiation. A metal halogen lamp (Osram-Ultramed 400 W) with an emission peak at 365 nm was used as the UV-A source. A UV-B filter consisting of 3 mm window glass was inserted between the lamp and the samples giving an output in UV-A of 10 mW/cm² sec at 30 cm distance as measured with a PUVA-meter (Waldmann AG, Schwenningen, GFR). For UV-B 2 fluorescent tubes (Westinghouse Sun Lamp, 40 W) emitting continuously from about 280 nm to 380 nm with a peak at 313 nm were used. These lamps have an output of 2.3 mW/cm² sec in the UV-B region as measured with a UV-meter (Waldmann AG, Schwenningen, GFR). For UV-C one fluorescent tube (Philips TUV 30 W) emitting at 254 nm was used, having an output of 2.1 mW/cm² sec as measured with a UVX digital radiometer (Ultra-violet products Inc, San Gabriel, Calif., USA).

Duplicate samples of drug and RBC, RBC only, as well as one sample containing drug only (the latter allowing compensation for possible UV induced colour changes of the drug), were irradiated and controls kept in the dark. After irradiation the samples were incubated in the dark. The volume of the samples was then measured to compensate for minor losses due to evaporation and the suspensions were transferred to test tubes and centrifuged for 10 min at 2000 rpm. Four ml of the supernatant was mixed with 1 ml of Drabkins' solution ($K_3Fe(CN)_6$ 200 mg, KNC 50 mg, KH_2PO_4 140 mg, surfactant Triton $\times$ 100 0.5 ml, diluted with distilled water to 1000 ml with a pH between 7.0 and 7.4) in order to convert all types of hemoglobin to methemoglobin which forms the stable pigment cyanmethemoglobin with a maximum absorbance at 540 nm. The samples were read at this wave length in a Beckman DB-GT spectrophotometer. A sample with 100% hemolysis consisted of 20 μ l of RBC in 12.5 ml of Drabkins' solution, also giving a dilution of 1:500.

Studies on the hemolytic effect of UV-irradiation without drug

The following UV-A doses were tested (corresponding doses in J/cm² in brackets): 1, 2, 3 and 4 h (36, 72, 108 and 144). Incubation time 2 h.

The following UV-B doses were tested (corresponding doses in J/cm² in brackets): 0.25, 0.5, 0.75, 1, 1.5, 2 and 4 h (2.1, 4.1, 6.2, 8.3, 12.4, 16.6 and 33.1). Incubation time 2 h.

When UV-C was tested physiological saline was used instead of Na-Veronal buffer as the latter was shown to absorb radiation at 254 nm. The following doses were tested (corresponding doses in J/cm² in brackets): 1, 3, 7, 15 and 30 minutes (0.1, 0.4, 0.9, 1.9 and 3.8).

Studies on the photohemolytic effect of DMCT

The influence of variations in incubation time, UV-A dose, DMCT concentration as well as the effect of UV-B were studied.

1. *Incubation time.* RBC in the presence of 100 μ g/ml of DMCT were irradiated for 2 h with UV-A. Samples were subsequently incubated for 0, 1, 2 and 3 h.

2. *UV-A dose.* RBC in the presence of 25 μ g/ml of DMCT were irradiated for 0.5, 1, 2 and 3 h. Incubation time 2 h.

3. *Concentration of DMCT.* RBC in the presence of DMCT 5, 10, 25, 50 and 100 μ g/ml were irradiated for 2 h with UV-A and incubated for 2 hours. This experiment was repeated at 10 different occasions to test the reproducibility of the method.

4. *Irradiation with UV-B.* RBC in the presence of DMCT 50 μ g/ml were irradiated with UV-B for 7, 15 and 30 min. RBC without DMCT, irradiated with the same doses, served as controls.

Studies on the photohemolytic effect of chlorpromazine (CPZ)

RBC in the presence of CPZ 0.1, 0.5, 1, 5 μ g/ml were irradiated for 2 h with UV-A and incubated for 2 h.

Studies on the effect of Drabkins' solution

RBC were irradiated with UV-B for 0.25, 0.5, 0.75, 1, 2 and 3 h without drug. For each UV-B dose four cups were used, two of which were treated with Drabkins' solution (as was the 100% control) according to the basic experimental design, the other two with 1 ml of buffer instead. For the latter two samples the 100% control was hemolysed with distilled water and not treated with Drabkin's

Hemolysis (%)

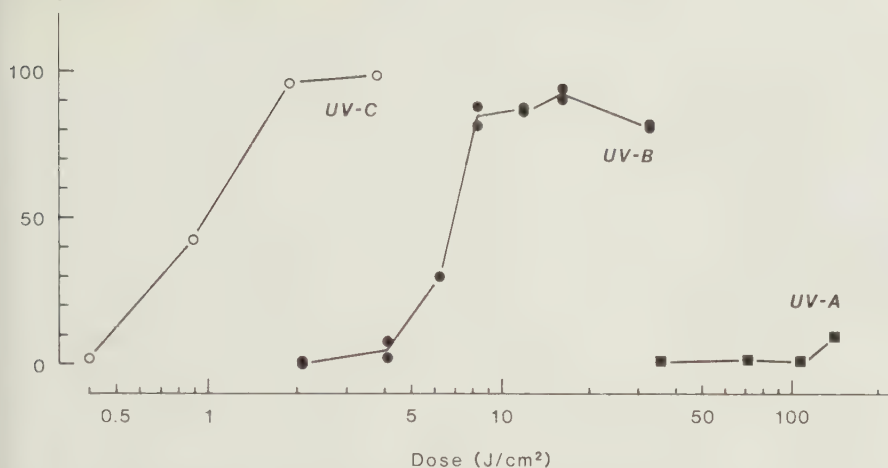


Fig. 1. Dose-response curves for photohemolysis. RBC without drug irradiated with increasing doses of UV-C, UV-B and UV-A respectively. Incubation time 2 h. The UV-B curve represents the mean of two experiments.

solution. This experiment was repeated irradiating RBC in 10, 25 and 100 µg/ml of DMCT for 2 h with UV-A. Incubation time 2 h.

Calculations

All results are expressed as per cent hemolysis relative to the 100% hemolysed solution using the following formula:

$$\frac{(EDR-ED) \times V-D}{T} \times 100$$

EDR = optical density of exposed drug solution with RBC

ED = optical density of exposed drug solution without RBC

D = optical density of dark control drug solution

T = optical density of total hemolysis control solution

V = $\frac{\text{volume after irradiation}}{\text{volume before irradiation}}$

RESULTS

Studies on the effect of UV-irradiation without drug

UV-A exposure. Only the longest exposure time of 4 h (144 J/cm²) induced a slight hemolysis of 10% (Fig. 1).

UV-B exposure. An exposure time of 0.5 h or less induced almost no hemolysis. Above this level an increasing rate of hemolysis was found reaching 91% after 2 h (16.6 J/cm²) (Fig. 1).

UV-C exposure. Hemolysis was induced with 7 min of irradiation and was total (99%) with 30 min of UV-C (3.8 J/cm²) (Fig. 1).

Studies on the photohemolytic effect of DMCT

1. **Incubation time.** For RBC UV-A irradiated for 2 h together with 100 µg/ml of DMCT a photohemolytic effect was shown. A 90% hemolysis rate was reached with 2 h incubation time which was therefore chosen as the standard in the following experiments (Fig. 2).

2. **UV-A dose.** When RBC were exposed to increasing UV-A doses in the presence of

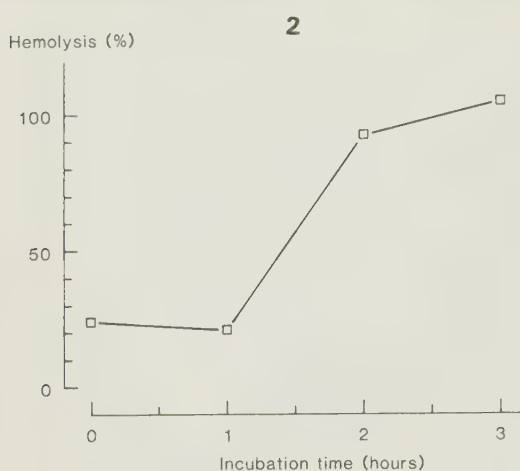


Fig. 2. Photohemolysis with different incubation times for RBC irradiated with 2 h of UV-A together with DMCT 200 µg/ml.

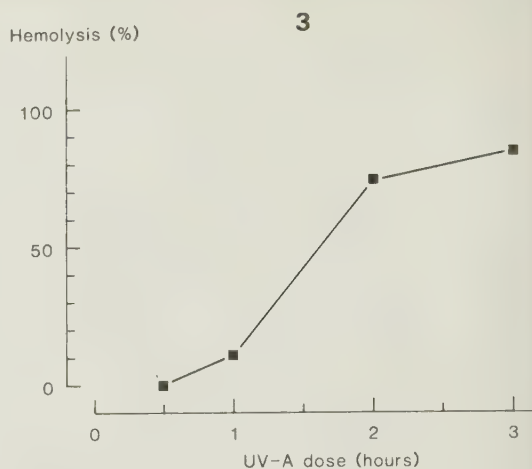


Fig. 3. Photohemolysis with DMCT. The effect of increasing UV-A doses for RBC in DMCT 25 µg/ml. Incubation time 2 h.

25 µg/ml DMCT a dose-response curve was obtained, levelling off above 2 h exposure. A maximum of 84% hemolysis was induced with 3 h of UV-A (Fig. 3). A standard exposure of 2 h (72 J/cm²) was used in the following experiments.

3. *Concentration of DMCT.* With 2 h of UV-A, 2 h incubation time and increasing concentrations of DMCT a dose-response curve was obtained with a maximal hemolysis of 88% at 50 µg/ml. The reproducibility was satisfying as indicated in Fig. 4.

4. *Irradiation with UV-B.* RBC irradiated with UV-B for 30 min (4.1 J/cm²) in the presence of DMCT 50 µg/ml showed no hemolysis.

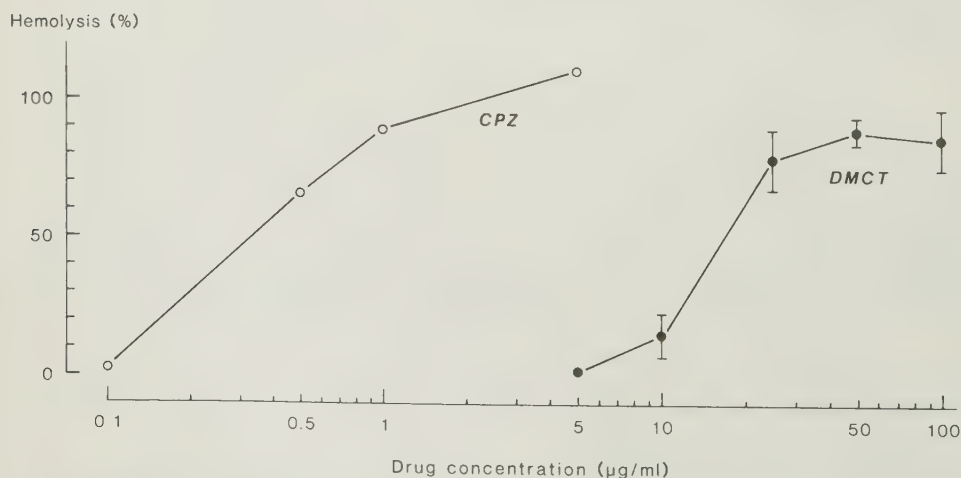


Fig. 4. Dose-response curves for CPZ and DMCT photohemolysis. RBC in increasing concentrations of CPZ and DMCT irradiated with 2 h of UV-A. Incubation time 2 h. The values for DMCT are the means of 10 experiments. Vertical bars indicate the standard deviation.

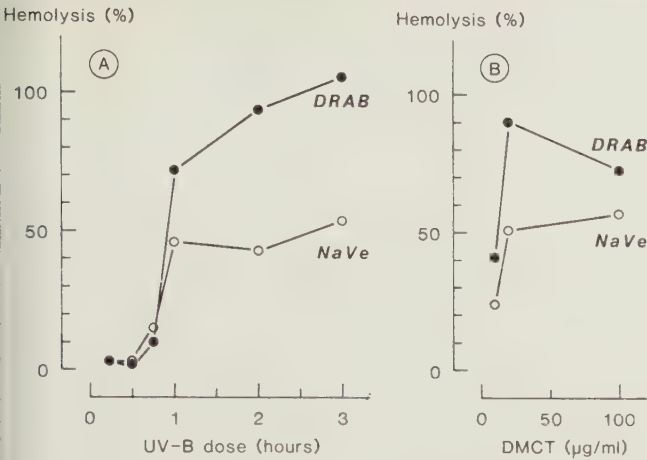


Fig. 5. Dose-response curves for photohemolysis showing the difference in hemoglobin determination with (DRAB) and without (NaVe) the use of Drabkins' method. A: RBC irradiated with UV-B without drug. Incubation time 2 h. B: RBC together with 10, 25 and 100 µg/ml of DMCT irradiated with 2 h of UV-A. Incubation time 2 h.

Studies on the photohemolytic effect of CPZ

CPZ had a very strong photohemolytic effect causing 89% hemolysis at 1 µg/ml and UV-A 2 h (Fig. 4).

Studies on the effect of Drabkins' solution

When RBC without drug were irradiated with UV-B for 1 h or more the addition of Drabkins' solution gave a higher value of hemolysis than the falsely low figure obtained with buffer alone. The difference increased with exposure time (Fig. 5 A). The difference after 1, 2 and 3 h of exposure was 26, 51 and 53% respectively. With exposures up to 0.45 h no difference was noted.

When RBC with DMCT 10, 25 and 100 µg/ml were irradiated with UV-A for 2 h the addition of Drabkins' solution gave higher values for all concentrations of DMCT. A maximal difference of 39% was estimated for DMCT 25 µg/ml (Fig. 5 B).

DISCUSSION

Exposure to UV-A alone had no hemolytic effect on RBC in doses below 108 J/cm². UV-B on the other hand caused marked hemolysis amounting to 91% at a dose of 16.6 J/cm². UV-C also showed a pronounced effect at even lower doses reaching 99% hemolysis at a dose of 3.8 J/cm². UV-C thus is at least 1000 times as potent as hemolyser than UV-A. The hemolytic ability of UV-B and UV-C has been reported earlier (11).

DMCT was shown to be a strong photosensitizer in combination with UV-A, hemolysis beginning at 10 µg/ml and reaching 88% at 50 µg/ml with 72 J/cm². A similar dose-response curve was obtained for increasing UV-A doses. The negative results in the UV-B experiments are in accordance with the action spectrum of DMCT, being in UV-A, as earlier reported (9). The reproducibility in repeated experiments was good. These findings support the data of Swanbeck & Wennersten (8) who also found DMCT to be photohemolytic in concentrations of 10 µg/ml or more combined with UV-A 132–264 J/cm². The negative results of Hasan et al. (10) may be due to the application of UV-A doses not greater than 20 J/cm². To further test the system CPZ was studied, giving hemolysis already at a concentration of 0.5 µg/ml, a rate of about fifty times that of DMCT.

It is important to compensate for possible colour changes of the irradiated drug solutions. UV-A exposure of DMCT causes a pronounced colour change with the photopro-

ducts absorbing at 540 nm which results in falsely high estimated hemolysis if not compensated for. This phenomenon was also observed by Kahn and Fleischaker (7).

Kahn & Fleischaker reported a phenomenon which they named the fading of hemoglobin (7). When irradiating RBC with UV-B and measuring the release of hemoglobin by reading the optical density at 540 nm they found a decreasing density with increasing exposure time. This was believed to reflect the conversion of some of the hemoglobin to methemoglobin with a new absorption peak at 630 nm. This problem can be overcome by the use of Drabkins' solution. By this method all forms of hemoglobin are converted to methemoglobin which forms a stable pigment with cyanide having an absorption maximum at 540 nm. We have shown the importance of this method in connection with UV-B exposure by finding a difference in hemolysis of 53% after 3 h of irradiation, the higher value obtained when Drabkins' solution was added. When testing Drabkins' solution in photohemolysis caused by DMCT and UV-A the difference was 39% at most.

In conclusion photohemolysis has proved to be a sensitive and accurate method for studying DMCT phototoxicity as well as the effects of ultraviolet radiation. Further studies including other tetracycline derivatives are in progress.

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Photohemolytic Potency of Tetracyclines*

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Hemolysis induced by long-wave ultraviolet radiation (UVA) and 8 different commercial tetracycline derivatives was studied in a model using human red blood cells. Demethylchlortetracycline and doxycycline were shown to have pronounced hemolytic properties causing 88% and 85% hemolysis, respectively, at a concentration of 50 $\mu\text{g/ml}$ and 72 J/cm^2 of UVA. Tetracycline, oxytetracycline, and chlortetracycline caused maximally 18% hemolysis at 200 $\mu\text{g/ml}$ and lymecycline only 7% at 100 $\mu\text{g/ml}$. Methacycline showed intermediate hemolytic effect of 36% at 200 $\mu\text{g/ml}$. Minocycline had no hemolytic effect whatsoever. These experimental data correlate very well with clinical reports and comparative phototoxicity trials in humans. Photohemolysis may thus be of value for predicting tetracycline phototoxicity.

It is well documented that the tetracyclines are able to induce light reactions in human skin and nails [1]. The incidence of skin photosensitivity following treatment with demethylchlortetracycline (DMCT) has been reported to be especially high but light reactions have also been reported with doxycycline, methacycline, oxytetracycline, chlortetracycline, and tetracycline hydrochloride [1]. To our knowledge there are no clinical reports about light-induced side effects with minocycline or lymecycline. In the majority of cases the reaction has been of the phototoxic type.

Studies have been performed to elucidate the phototoxic potential of different tetracyclines. In these studies human volunteers have been given one of the tetracycline derivatives prior to natural sunlight exposure and examined for abnormal sunburn reactions. Blank et al, comparing DMCT and doxycycline, thus found 9 out of 10 and 2 out of 10 individuals, respectively, with abnormal reactions [2]. Frost et al, studying DMCT vs methacycline, found 12 out of 13 and 1 out of 14, respectively, with phototoxic reactions [3]. The same investigators, comparing doxycycline and minocycline, noted 11 out of 15 and none out of 19, respectively, with abnormal reactions [4].

Different experimental models have also been used for studying tetracycline phototoxicity. We have recently shown photohemolysis to be an accurate method for studying DMCT phototoxicity provided large enough doses of long-wave ultraviolet radiation (UVA) are applied [5]. The present study was designed to compare the photohemolytic capacity of other tetracycline derivatives and to relate this to clinical reports on phototoxic properties.

MATERIALS AND METHODS

Drugs

The 8 commercial tetracycline derivatives were provided by the following companies: demethylchlortetracycline, tetracycline, chlortetracycline, and minocycline by Lederle Laboratories, Pearl River, New York; doxycycline, oxytetracycline, and methacycline by Pfizer, Brussels, Belgium; and lymecycline by Farmitalia Carlo Erba, Taby, Sweden.

Basic Experimental Design

The photohemolysis technique described by Kahn and Fleischaker [6], recently modified by us [5], was used.

Red blood cells (RBC) were obtained by venipuncture from healthy human volunteers not taking any drugs. RBC were drawn into ACD and then immediately washed in physiologic saline and centrifuged for 10 min at 2000 rpm. This procedure was repeated 3 times, whereafter the RBC were stored refrigerated no longer than 40 h before use to avoid spontaneous hemolysis.

The test drug was dissolved in a buffer solution consisting of 0.075 M Na-Veronal (barbital) at pH 7.4 with saline to 290 mosmol/kg. Ten milliliters of the buffered drug solution was poured into plastic cups forming a liquid layer of 7 mm. RBC 20 μl were added to each cup, giving a dilution of 1:500. The cups were then irradiated with long-wave (UVA) or medium-wave (UVB) ultraviolet radiation. A metal halogen lamp (Osram-Ultramed 400 W) with an emission peak at 365 nm was used as the UVA source. A UVB filter consisting of 3-mm window glass was inserted between the lamp and the samples, giving an output in UVA of 10 mW/cm^2 s at 30-cm distance as measured with a PUVA-meter (Waldmann AG, Schwenningen, G.F.R.). For UVB 2 fluorescent tubes (FS 40 Westinghouse Sun Lamp, 40 W) emitting continuously from 280–380 nm with a peak at 313 nm were used, giving an output of 2.3 mW/cm^2 s in the UVB region as measured with a UV-meter (Waldmann AG).

Duplicate samples of drug and RBC, RBC only, as well as 1 sample containing drug only (the last allowing compensation for possible UV-induced color changes of the drug) were irradiated and controls kept in the dark. After irradiation the samples were incubated in the dark. The volume of the samples was then measured to compensate for minor losses due to evaporation and the suspensions were transferred to test tubes and centrifuged for 10 min at 2000 rpm. Four milliliters of the supernatant was mixed with 1 ml of Drabkins' solution [$\text{K}_2\text{Fe}(\text{CN})_6$ 200 mg, KNC 50 mg, KH_2PO_4 140 mg, surfactant Triton X-100 0.5 ml, diluted with distilled water to 1000 ml with pH 7.0–7.4] in order to convert all types of hemoglobin to methemoglobin which forms the stable pigment cyanmethemoglobin with a maximum absorbance at 540 nm [7]. This procedure further dilutes the hemoglobin to 1:625. The samples were read at 540 nm in a Beckman DB-GT spectrophotometer. A sample with 100% hemolysis consisted of 20 μl of RBC in 12.5 ml of Drabkins' solution, also giving a dilution of 1:625.

Studies on the Photohemolytic Effect of Tetracyclines and UVA

RBC in the presence of DMCT and doxycycline 5, 10, 25, 50, and 100 $\mu\text{g/ml}$, and for the other derivatives 10, 25, 50, 100, and 200 $\mu\text{g/ml}$, were irradiated with UVA for 2 h (72 J/cm^2) and dark incubated for 2 h. For DMCT the experiment was repeated 10 times and for the other substances at least twice on different occasions.

Studies on Irradiation with UVB

RBC in the presence of DMCT 50 $\mu\text{g/ml}$, methacycline 200 $\mu\text{g/ml}$, and oxytetracycline 200 $\mu\text{g/ml}$ were irradiated with UVB 7, 15, and 30 min (1, 2.1, and 4.1 J/cm^2). These UVB doses had earlier been shown to have no hemolytic effect [5].

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Reprint requests to: Mats Bjellerup, M.D., Lund University, General Hospital, S-214 01 Malmö, Sweden.

Abbreviations:

DMCT: demethylchlortetracycline

RBC: red blood cells

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Studies on Preirradiation with UVA

RBC were incubated in the dark for 3 h with DMCT or doxycycline 5, 10, 25, 50, and 100 $\mu\text{g/ml}$ preirradiated for 2 h with UVA.

Calculations

All results are expressed as % hemolysis relative to the 100% hemolysed solution using the following formula:

$$\frac{(EDR - ED)V - D}{T} \times 100$$

where EDR = optical density of exposed drug solution with RBC

ED = optical density of exposed drug solution without RBC

D = optical density of dark control drug solution

T = optical density of total hemolysis control solution

$$V = \frac{\text{volume after irradiation}}{\text{volume before irradiation}}$$

For statistical evaluation Student's *t*-test was used.

RESULTS

Studies on the Photohemolytic Effect of Tetracycline and UVA

DMCT and doxycycline showed pronounced hemolytic properties with a maximum of 88% and 85% hemolysis, respectively, at a concentration of 50 $\mu\text{g/ml}$ (Fig 1). A dose-response curve leveling off above 50 $\mu\text{g/ml}$ was obtained. The reproducibility of the results was good as shown in Fig 1. Methacycline showed intermediate hemolytic effect of 36% at 200 $\mu\text{g/ml}$. Tetracycline, oxytetracycline, and chlortetracycline caused hemolysis of maximally 18% at 200 $\mu\text{g/ml}$ and lymecycline of only 7% at 100 $\mu\text{g/ml}$. Minocycline had no hemolytic effect whatsoever. The hemolytic effect of DMCT was significantly stronger ($p < 0.001$) than for the other tetracyclines, except doxycycline.

Studies on Irradiation with UVB

RBC in the presence of DMCT, methacycline, and oxytetracycline irradiated with doses of UVB previously shown to be subhemolytic caused no hemolysis.

Studies on Preirradiation with UVA

RBC incubated with preirradiated DMCT and doxycycline induced no hemolysis.

Hemolysis (%)

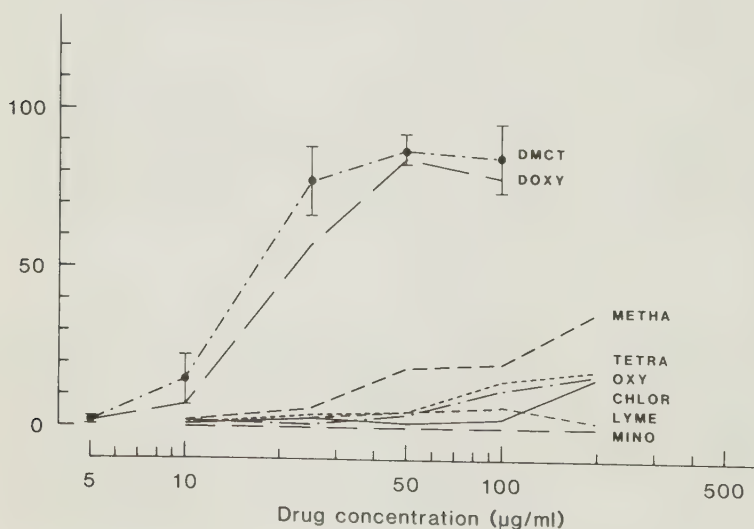


FIG 1. Dose-response curves for photohemolysis with DMCT, doxycycline, methacycline, tetracycline, oxytetracycline, chlortetracycline, lymecycline, and minocycline. RBC in increasing concentrations of drug were irradiated for 2 h with UVA and dark incubated for 2 h. The values for DMCT are the mean of 10 experiments, vertical bars indicating the SD.

DISCUSSION

The different tetracyclines thus have varying photohemolytic properties. DMCT and doxycycline were strong hemolysers under the present conditions; methacycline intermediate; tetracycline, oxytetracycline, chlortetracycline, and especially lymecycline were weak hemolysers; whereas minocycline showed no photohemolytic effect whatsoever. This corresponds fairly well with another experimental *in vivo* model measuring phototoxic edema in the mouse tail. Ljunggren and Möller, testing the same tetracyclines, thus found DMCT, doxycycline, and to a lesser degree lymecycline to have phototoxic effects [8].

RBC in the presence of DMCT, methacycline, and oxytetracycline irradiated with UVB showed no hemolysis. This indicates that the action spectrum is in the UVA as earlier reported in humans by Schorr and Monash [9].

RBC incubated with preirradiated DMCT and doxycycline showed no hemolysis, indicating no formation of toxic photoproducts, which is the case in photohemolysis due to chlorpromazine as reported by Johnson [10].

The present photohemolytic data correspond very well with clinical reports on phototoxicity in humans [1]. These data are also in accordance with the few comparative trials between different tetracyclines that have been performed in humans [2-4]. It should be pointed out that the concentrations of DMCT and doxycycline needed to achieve total hemolysis (25-50 $\mu\text{g/ml}$) are much higher than those found in human serum under therapeutic conditions (2-3 $\mu\text{g/ml}$). The UVA dose given (72 J/cm^2) also is higher than under natural conditions. In another model using cultured human fibroblasts, however, we have found UVA doses as small as 1.9 J/cm^2 to be phototoxic in combination with 10 $\mu\text{g/ml}$ of DMCT or doxycycline (unpublished data). Under natural conditions a photoaugmentative mechanism also may be of importance, UVB augmenting the phototoxic reaction caused by UVA and tetracycline, as has been described in mice [11].

Photohemolysis thus is an accurate method for studying tetracycline phototoxicity. In view of the good correlation with clinical reports and comparative trials in humans it may be of value as a predictive test when new tetracyclines are introduced.

We wish to thank Mrs. Karin Lundberg for skillful technical assistance.

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Influence of Tetracycline Phototoxicity on the Growth of Cultured Human Fibroblasts

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The phototoxic effect of 8 different commercial tetracycline derivatives with long-wave ultraviolet radiation (UVA) on the growth pattern of normal human skin fibroblasts in culture was studied. Chlortetracycline and doxycycline both at a concentration of 50 $\mu\text{g}/\text{ml}$ and 1.9 J/cm^2 of UVA resulted in total cell death with no recovery during a 14-day observation period. Demethylchlortetracycline also showed strong photosensitizing properties with an arrested cell division for 7 days followed by a recurrence of cell growth. The other tetracyclines tested under identical conditions had only weak or no phototoxic influence on cell growth. These experimental data correlate very well with clinical reports and comparative phototoxicity trials in humans. This experimental method may thus be of value for predicting tetracycline phototoxicity in humans.

It is well documented that the tetracyclines are able to induce light reactions in human skin and nails [1]. The incidence of skin photosensitivity following treatment with demethylchlortetracycline (DMCT) has been reported to be especially high [1]. To our knowledge there are no clinical reports about light-induced side effects from minocycline. In 3 studies on tetracycline phototoxic potential, human volunteers have been given one of the tetracycline derivatives prior to natural sunlight exposure and examined for abnormal reactions. Blank et al [2], comparing DMCT and doxycycline, thus found 9/10 and 2/10 individuals, respectively, with abnormal reactions. Frost et al [3], studying DMCT vs methacycline, found 12/13 and 1/14, respectively, with phototoxic reactions [3]. The latter investigators, comparing doxycycline and minocycline, noted 11/15 and 0/19, respectively, with abnormal reactions [4].

Different experimental models have also been used for studying tetracycline phototoxicity. In a recent study, using a photohemolysis technique, we showed that DMCT and doxycycline had pronounced hemolytic properties [5]. Since human erythrocytes are cells without nuclei we found it of interest to expand the studies to cultured human skin fibroblasts which are nucleated cells in division.

MATERIALS AND METHODS

Drugs

The 8 commercial tetracycline derivatives were provided by the following companies: DMCT, tetracycline, chlortetracycline, and minocycline by Lederle Laboratories, Pearl River, New York; doxycycline, oxytetracycline, and methacycline by Pfizer, Brussels, Belgium; and lymecycline by Farmitalia Carlo Erba, Taby, Sweden.

Basic Experimental Design

Fibroblasts were obtained and cultured as described earlier [6]. Skin biopsies from 8 healthy males aged 13-75 years (mean 48.7) were cut

into small pieces, and placed at the bottom of 25-cm² plastic culture flasks (A/S Nunc, Denmark). The flasks were turned upside down and 5 ml medium was added. The biopsies were left uncovered with medium for 1-1.5 h to ensure adhesion before the flasks were turned again to be left untouched during 10 days at 37°C for outgrowth. All medium components were obtained from Gibco, Chemoferm AB, Sweden. Minimum essential medium with Hanks' salts was supplemented with heat-inactivated fetal calf serum (10%), L-glutamine (585 mg/liter), nonessential amino acid mixture (1%), and gentamicin (100 mg/liter). During outgrowth medium was changed twice a week. Confluent cells were subcultivated by the use of 0.05% trypsin-0.02% EDTA.

For the experiments confluent cells from passages 1-6 were used. During irradiation of the flasks medium was replaced by drug dissolved in physiological saline adjusted to pH 7.2 with NaOH. Nonirradiated drug-exposed cell cultures were kept in the dark for equally long periods as controls. Controls that were neither irradiated nor treated with drug were kept in the dark in physiologic saline.

The cells were irradiated in the flasks at 10 cm distance with 2.5-20 min long-wave ultraviolet radiation (UVA) from 2 blacklight fluorescent tubes (Philips TLA 40 W/08) with an emission peak at 360 nm. A 3-mm window glass filter was inserted to eliminate wavelengths shorter than 320 nm. The output inside the flask at the level of the cells was 2.1 mW/cm² in the UVA as measured with a PUVA-meter (Waldmann AG, Schwenningen, G.F.R.).

After irradiation the drug solution was poured off and the cells in each flask were trypsinized, counted in a Bürker chamber, and subcultivated into a tissue culture multiwell plate containing 12 wells with an area of 4.5 cm² (Linbro, Linbro Scientific, Inc., Hamden, Connecticut). The initial cell density of a control well was approximately 1.0×10^5 cells per well. The cells were then left to grow and were counted on days 1, 3, 7, 10, 14, and sometimes 21 with 2 wells counted on each occasion.

All experiments comparing different drugs were performed simultaneously with cells from one donor.

Studies on Doxycycline

Fibroblasts in the presence of doxycycline 5, 10, 25, and 50 $\mu\text{g}/\text{ml}$ were irradiated with 1.9 J/cm^2 (15 min) of UVA. In a second experiment fibroblasts in the presence of doxycycline 50 $\mu\text{g}/\text{ml}$ were irradiated with 0.3, 0.6, 1.3, and 1.9 J/cm^2 of UVA (2.5, 5, 10, and 15 min). As control, cells with no drug added were irradiated with 2.5 J/cm^2 of UVA (20 min).

Studies on the Phototoxic Potency of Eight Different Tetracycline Derivatives

Fibroblasts in the presence of 50 $\mu\text{g}/\text{ml}$ of each of the tetracycline derivatives were irradiated with 1.9 J/cm^2 of UVA. This experiment was repeated with fibroblasts from another donor. With DMCT alone the experiment was repeated on 4 different occasions.

RESULTS

Studies on Doxycycline

A dose-response relationship concerning growth inhibition was obtained with increasing concentrations of doxycycline resulting in total cell death with 25 and 50 $\mu\text{g}/\text{ml}$ (Fig 1A). A dose-response curve was also obtained with a constant doxycycline concentration (50 $\mu\text{g}/\text{ml}$) but increasing doses of UVA with a total cell death at 1.9 J/cm^2 (Fig 1B). UVA irradiation of 2.5 J/cm^2 did not affect cell growth.

Studies on the Phototoxic Potency of Eight Different Tetracycline Derivatives

As can be seen from Fig 2, chlortetracycline and doxycycline addition both resulted in a cell number decreasing to almost

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Abbreviations:

DMCT: demethylchlortetracycline

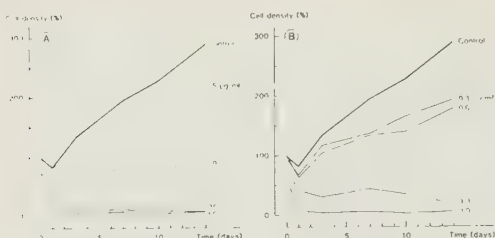


FIG 1. Cell growth pattern for fibroblasts treated with doxycycline 5, 10, 25, and 50 $\mu\text{g/ml}$ combined with UVA 1.9 J/cm^2 (A) or doxycycline 50 $\mu\text{g/ml}$ combined with UVA 0.3, 0.6, 1.3, and 1.9 J/cm^2 (B) on day 0 compared to untreated cells. Cell density of control cells immediately after irradiation on day 0 is plotted as 100% and serves as baseline control for all curves in this experiment.

zero on day 3 with no tendency to recovery during 14 days' observation. DMCT supplementation resulted in a temporary arrest in cell growth, during 7 days, whereafter normal cell growth recurred. The other tetracyclines tested had only weak or no phototoxic influence on cell growth at the concentration tested. A second experiment with all 8 derivatives, as well as 4 experiments with DMCT alone, showed similar results. In no case did tetracycline derivatives themselves, i.e., without UVA, affect cell growth.

DISCUSSION

Doxycycline and chlortetracycline followed by DMCT were the strongest photosensitizers in this study on normal human skin fibroblasts. The other derivatives did not significantly influence cell growth. These findings closely correlate with the results of Hasan et al studying decrease of [^3H]thymidine incorporation into human lymphocytes [7]. The correlation also is fairly good between our results in fibroblasts and a recent *in vitro* study using photohemolysis [5]. Investigations on dividing fibroblasts may constitute a more physiologic system than the use of erythrocytes, which are devoid of nuclei and without the capacity to divide. This is also indicated by the lower and more physiologic UVA doses applied in the present study. It should also be pointed out that doxycycline affected cell growth in as low concentrations as 5–10 $\mu\text{g/ml}$ which is in the same order of magnitude as the normal serum concentration in humans (2–4 $\mu\text{g/ml}$) [8].

Does the phototoxic potency of the different tetracycline derivatives in cultured human skin fibroblasts parallel their clinical photosensitizing capacity in humans? DMCT is the drug most frequently occurring in scattered clinical reports [1] and also it had great sensitizing capacity in 2 comparative trials using human volunteers [2,3]. Chlortetracycline comes second concerning reported clinical photoreactions [1], but unfortunately there are no comparative trials including this drug. Doxycycline (the most prescribed tetracycline in Sweden but rarely used in the United States) is the tetracycline most frequently causing photoreactions as reported to the Drug Information Committee, Swedish National Board of Health

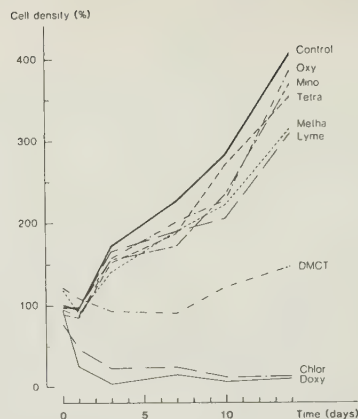


FIG 2. Cell growth pattern for fibroblasts treated with oxytetracycline, minocycline, tetracycline, methacycline, lymecycline, DMCT, chlortetracycline, and doxycycline 50 $\mu\text{g/ml}$ and UVA 1.9 J/cm^2 on day 0 compared to untreated cells. For explanation see Fig 1.

and Welfare [9]. This drug also has shown medium to strong photosensitizing capacity in comparative trials [2,4]. The other tetracycline derivatives, which demonstrated only weak phototoxic properties in this study, are rarely reported clinically as sensitizers [1] and are negative or weak (minocycline and methacycline, respectively) phototoxic agents in existing comparative trials [3,4].

There thus seems to be a good correlation between this experimental study and clinical conditions.

We wish to thank Mrs. Kerstin Knutsson and Mrs. Karin Lundberg for skilful technical assistance.

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IV:I

DOUBLE BLIND CROSS-OVER STUDIES ON PHOTOTOXICITY TO
THREE TETRACYCLINE DERIVATIVES IN HUMAN VOLUNTEERS

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ABSTRACT

A double blind cross-over phototoxicity study using demethylchlortetracycline (DMCT) 0.3 g x 2, doxycycline 0.1 g x 2, lymecycline 0.6 g x 2 and placebo was performed on 8 healthy human volunteers. Drugs were given for 3 consecutive days and on the third day the volunteers were tested with different modalities of artificial longwave ultraviolet radiation (UVA) and assessed 24 h later for objective as well as subjective abnormal photoreactions. All four substances were tested in each individual at weekly intervals and serum concentrations of tetracycline were determined. Taking all objective and subjective reactions into account 2/8 reacted to lymecycline, 2/8 to DMCT and 7/8 to doxycycline. There was no significant difference in serum concentration between the three derivatives. It is concluded that doxycycline is the most potent photosensitizer at the dosage chosen.

Introduction

The tetracyclines are well known photosensitizers in clinical work (1). The reactions are of the phototoxic type and appear clinically as an exaggerated sunburn sometimes with edema and blistering. A small proportion of the patients with skin photosensitivity also develops photoonycholysis. There are differences in phototoxic potency between tetracycline derivatives as shown by experiments in vitro as well as clinical investigations.

In the present investigation the ability to induce clinical phototoxicity in healthy volunteers was studied for doxycycline, lymecycline and DMCT. Doxycycline and lymecycline were chosen because they are the two most prescribed tetracyclines on the market (74% and 14% respectively of total daily doses prescribed in Sweden 1985) and were given in maximum recommended doses (twice the standard dose). Lymecycline has recently been shown to be a "pro-drug" dissociating in the gastro-intestinal tract to yield tetracycline. It can be detected as such in plasma (2). DMCT was included for comparison as it is the tetracycline most often reported to cause phototoxicity in the literature, but little used in Sweden (0.003% of daily doses prescribed), and was given at recommended dose. Each person was tested with all three derivatives and placebo. Different modalities of UVA irradiation were used and objective as well as subjective reactions were recorded. Serum levels of tetracycline were analysed.

MATERIALS AND METHODS

Volunteers

Eight healthy volunteers (7 females and 1 male, 21-44 yr, 6 with skin type II and 2 with skin type III), from whom informed consent had been obtained, participated in the study. None of the test persons had received any systemic medication for at least 5 days before the start of the study.

Drugs

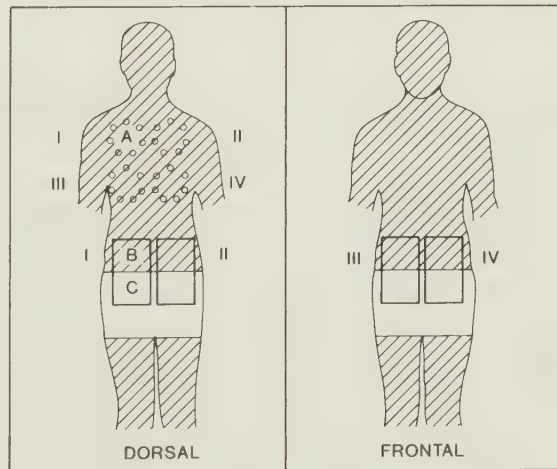
The three commercial tetracycline derivatives tested were: Demethylchlor-tetracycline (Ledermycin^R), Lederle Laboratories, Pearl River, New York; doxycycline (Vibramycin^R), Pfizer, Brussels, Belgium; and lymecycline (Tetralysal^R), Farmitalia Carlo Erba, Täby, Sweden. Placebo tablets containing cellulose and lactose were obtained from the hospital pharmacy. All medication was given for three days, DMCT at the recommended dosage 0.3 g twice daily, doxycycline and lymecycline at the highest recommended dosage (0.1 g twice daily and 0.6 g twice daily respectively).

Placebo drug was given as 2 tablets twice daily. All four preparations were tested randomly in a double blind cross-over fashion in each individual with a wash-out period of 4 days minimum.

Phototesting

Phototesting was done two hours after the last drug dose on day 3. For each drug two modalities of UVA were tested according to fig 1; (i) on the back 7 areas, measuring 3 cm^2 each, were irradiated with increasing doses of UVA, (1.2 - 20.3 J) with an Osram Ultramed^R 400 W metal halogen lamp mounted in a Blue-Light-Spot^R housing equipped with filter h 1 (dr Hönle, GmbH, Munich, West Germany) emitting continuously from 320 nm with a peak at 370 nm. The output at the level of the skin was 26 mW/cm^2 sec in the UVA range as measured with a UVX digital radiometer (Ultraviolet Products Inc, San Gabriel, California, USA); (ii) an area measuring $12 \times 20 \text{ cm}$ of previously sunexposed skin on the lower back or abdomen was irradiated with 20 J of UVA with a PUVA 4000 - equipment (Waldman AG, Schwenningen, West Germany) emitting mainly in the UVA region with a peak at 365 nm; (iii) an area measuring $10 \times 12 \text{ cm}$ of previously unexposed skin on the lower back or abdomen was also irradiated with 20 J of UVA in the PUVA 4000 - equipment. The tests were read blindly 24 hours later by one of the investigators (MB) and the test persons were questioned about subjective side effects particularly the sensation of stinging. Skin reactions were graded as 1 (erythema), 2 (strong erythema) and 3 (erythema and edema). Stinging was graded as 1 (mild) and 2 (severe).

Fig 1. Experimental design showing 2 different phototesting modalities: A, graded doses of UVA 1.2-30.2 J; B, single dose of UVA 20 J given to sun-exposed skin; C, single dose of UVA 20 J given to unexposed skin. Phototesting was performed on 4 different occasions (I-IV) with either DMCT, doxycycline, lymecycline or placebo.



Tetracycline serum levels

Immediately before phototesting a 5 ml blood sample was drawn, the serum separated and kept at -22°C until analysed for tetracycline. A spectrophotofluorometric method for measuring tetracycline routinely used at the Department of Toxicology, and described elsewhere (3), was used. When measuring serum levels in persons taking lymecycline, tetracycline was used as standard.

Statistics

For statistical analysis the Student's t-test and Wilcoxon rank sum test were used.

RESULTS

Skin reactions with graded UVA doses (1.2 - 20.3 J)

Erythema scores with graded UVA doses were added in each subject for the different drugs (table 1). Reactions stronger than those with placebo developed in 3/8 persons taking DMCT, 6/8 taking doxycycline and 5/8 taking lymecycline. Comparing the drugs, however, must be done with caution since UVA in itself, combined with placebo, produced erythema exceeding that produced by one or more of the tetracycline derivatives and UVA in some persons. Differences between drugs are statistically not significant (Wilcoxon rank sum test).

Table I. Erythema scores added in each individual with graded doses of UVA (1.2-20.3 J) in combination with placebo (plac), demethylchlor-tetracycline (DMCT), doxycycline (doxy) and lymecycline (lyme). Reactions judged clinically relevant are printed in bold figures.

Individual no.	Placebo	DMCT	Doxy	Lyme
1.	2.5	-	3	3
2.	0.5	-	2	2.5
3.	-	0.5	1.5	2
4.	4	1.5	2	1
5.	1	1	4	-
6.	1	0.5	2	1
7.	0.5	8	1.5	5
8.	2	3	0.5	3
Summary	11.5	14.5	16.5	17.5

Skin reactions with 20 J of UVA to large areas of previously exposed and non-exposed skin

In 5 of the subjects UVA (combined with placebo) produced erythema in itself, making weak reactions with tetracycline and UVA of no significance (table II). However, two strong reactions were noted, both with doxycycline and in unexposed skin. Differences between drugs are statistically not significant (Wilcoxon rank sum test).

Table II. Erythemat reactions to a single dose of UVA (20J) to large areas of previously exposed (exp) and unexposed (non-exp) skin. For further information see table I.

Individual no.	Placebo		DMCT		Doxy		Lyme	
	Exp.	Non-exp.	Exp.	Non-exp.	Exp.	Non-exp.	Exp.	Non-exp.
1.	-	-	-	-	-	-	0.5	0.5
2.	1	1	1	1	1	3	1	2
3.	-	-	0.5	1	-	-	0.5	0.5
4.	-	-	-	0.5	-	-	-	-
5.	1	1.5	1	1	1	1	1	2
6.	1	1	1	1	1	3	1	1
7.	1	1	0.5	0.5	-	0.5	0.5	0.5
8.	1.5	2	1	2	1	2	2	2

Subjective reactions

When graded doses of UVA were tested a sensation of stinging, exceeding that with UVA and placebo, was recorded in 2/8 persons with DMCT, 4/8 with doxycycline and 2/8 with lymecycline (table III). When a single dose of UVA was given to a large area stinging was never recorded with placebo, DMCT or lymecycline as opposed to doxycycline which produced this effect in 3/8 subjects (table III). Adding all scores, no statistically significant differences between the groups could be shown (Wilcoxon rank sum test).

Added objective and subjective reactions for both radiation modalities

An attempt to illustrate substantial reactions specified for each individual is shown in table IV. With graded doses of UVA a reaction has been judged significant when it exceeds the corrected placebo score (placebo score + negative deviation (when existing) for any of the other two tetracyclines compared to placebo) with > 2 units. With a single dose of UVA a reaction was judged significant if it exceeded placebo with ≥ 2 units. The effect of stinging was judged significant when it exceeded placebo reactions. Original data, in this way judged significant, are emphasized by bold figures in table I - III. Using this method of ranking, reactions of any kind were recorded in 7/8 provocations with doxycycline and 2/8 with DMCT and lymecycline.

Individual no.	Placebo			DMCT			Doxy			Lyme		
	UVA			UVA			UVA			UVA		
	Single		Graded	Single		Graded	Single		Graded	Single		Graded
	Exp.	Non-exp.		Exp.	Non-exp.		Exp.	Non-exp.		Exp.	Non-exp.	
1.	-	-	-	-	-	-	-	-	2	-	-	1
2.	-	-	1	-	-	-	-	-	-	-	-	1
3.	-	-	-	-	-	1	1	1	1	-	-	-
4.	-	-	-	-	-	-	-	-	-	-	-	-
5.	-	-	1	-	-	1	-	-	1	-	-	1
6.	-	-	-	-	-	-	1	1	1	-	-	-
7.	-	-	-	-	-	2	-	-	1	-	-	1.5
8.	-	-	2	-	-	1	1	1	1	-	-	1.5

Table III. Subjective reactions with a single dose of UVA to previously exposed (exp) and unexposed (non-exp) skin and with graded doses of UVA. For further explanation see table I.

		Individual no.							
		1.	2.	3.	4.	5.	6.	7.	8.
DMCT	Serum conc.	4.0	1.1	2.5	3.2	1.9	4.2	8.1	3.6
	Graded UVA Obj. Subj.			+				+	
	Single UVA Obj. Subj.								
Doxy	Serum conc.	3.2	4.2	5.7	3.8	0.9	2.1	0.9	1.6
	Graded UVA Obj. Subj.	+		+		+	+	+	
	Single UVA Obj. Subj.		+	+			+		+
Lyme	Serum conc.	8.5	3.5	4.4	6.0	1.1	2.3	2.0	2.0
	Graded UVA Obj. Subj.	+						+	
	Single UVA Obj. Subj.								

Table IV. Added objective and subjective reactions judged clinically relevant with graded and single doses of UVA. Serum levels of tetracycline are included (microgram/ml). For further explanation see table I - III.

Tetracycline serum levels

Individual serum values are shown in table IV. In no case was any remaining tetracycline detectable when the test person was on placebo, indicating that the chosen wash-out period was sufficient. The following tetracycline levels (standard deviation in brackets) were recorded; DMCT 3.6 ($\pm$ 2.1) microgram/ml, doxycycline 2.8 ($\pm$ 1.7) microgram/ml and lymecycline (measured as tetracycline) 3.7 ($\pm$ 2.5) microgram/ml. There was no statistically significant difference between the three derivatives using Student's t-test. Statistical analysis of serum levels in each subject showed no tendency of high serum levels for all derivatives to occur in the same person (Wilcoxon rank sum test). There was no clear cut correlation between reactivity and high serum levels.

DISCUSSION

Experimental in vitro studies on erythrocytes (4), fibroblasts (5), lymphocytes (6) and bacteriophage DNA (7) have shown doxycycline, DMCT and chlortetracycline to be the most photoactive compounds. Tetracycline, oxytetracycline, methacycline and lymecycline were weak photosensitizers while minocycline had no phototoxic effect whatsoever. In mice, DMCT and doxycycline were the most potent sensitizers (8).

Do these experimental data parallel the clinical photosensitizing capacity in humans?

Three comparative studies have been performed previously. Human volunteers were given one of the tetracycline derivatives orally prior to natural sunlight exposure and examined for abnormal reactions. Blank et al (9) comparing DMCT (0.6 g daily) and doxycycline (0.2 g daily), thus found 9/10 and 2/10 individuals respectively with abnormal reactions. Frost et al (10), studying DMCT (0.6 g daily) versus methacycline (0.6 g daily), found 12/13 and 1/14 respectively with phototoxic reactions. The latter investigators, comparing doxycycline (0.2 g daily) and minocycline (0.2 g daily), noted 11/15 and 0/19 respectively with abnormal reactions (11). For further comparative evaluation only scattered clinical reports exist. DMCT is the drug most frequently involved in such reports (1) and, as mentioned, DMCT also had great photosensitizing capacity in the two comparative trials on human volunteers (9, 10). Chlortetracycline comes second concerning reported clinical photoreactions (1), but unfortunately there are no comparative trials including this drug.

In Sweden doxycycline is the tetracycline most frequently causing photoreactions as reported to the Drug Information Committee (12).

This drug also shows medium to strong photosensitizing capacity in comparative trials (9, 11). The other tetracycline derivatives are rarely reported clinically as sensitizers and are negative or weak (minocycline and methacycline respectively) phototoxic agents in existing comparative trials (10, 11). There thus seems to be a good correlation between in vitro experimental studies and clinical conditions.

The present data speak in favour of doxycycline being a more potent photosensitizer than lymecycline. Concerning the comparatively few reactions to DMCT it should be noted that this drug, which was included as the best known photosensitizer of the tetracyclines, was given in the recommended standard dose only, while the two others were given in double the recommended standard dose. Lymecycline caused reactions in a few individuals only, and it was never the sole reacting substance as was the case with doxycycline in 4 persons (table IV).

Phototoxicity to tetracyclines is difficult to produce clinically in comparison with, for example, 8-methoxypsoralen (13). Especially does this seem to be the case when artificial UV-light is used. Thus Rosén and Swanbeck using a high intensity UVA lamp could demonstrate increased sensitivity to UVA in only 1/10 persons taking doxycycline (0.1 g daily) (14). In pilot experiments with doxycycline 0.1 g daily and lymecycline 0.6 g daily we were unable to detect phototoxicity (as erythema reactions) in 10 volunteers using artificial high intensity UVA (unpublished observations). Similar observations have been made in chlortetracycline phototoxicity where a group of children suffered a clear relapse with natural sunlight but not when tested with an artificial light source (15). In the mouse, doxycycline phototoxicity elicited by UVA is strongly augmented by the addition of UVB indicating the importance of this type of radiation as a cofactor (16). As has been shown in the present study reactions are more easily detected if subjective symptoms are also recorded. This was also demonstrated in volunteers taking doxycycline and exposed to natural sunlight (11).

Theoretically, photoreactions of the phototoxic type, should be possible to provoke in all individuals provided that the tetracycline concentration and UVA dose delivered to the target organ are sufficiently large.

The fact that not all individuals reacted, as well as the lack of correlation between tetracycline serum levels and photoreactivity in this study (such correlation has been shown in another study (11)), indicate that individual factors may be of major importance. As has been demonstrated in this study the tetracycline derivatives produced different serum levels in one and the same individual and the levels did not correlate with photoreactivity. This fact, however, does not exclude a reciprocal relationship between serum levels and photoreactivity in a single individual. In a study on 8-methoxypsoralen, there was a clear cut reciprocal relationship between dose, serum levels and photoreactivity within each individual (13).

In conclusion, the phototoxic potential of DMCT, doxycycline and lymecycline has been verified using artificial UV-light. Testing lymecycline and doxycycline in comparable doses, the latter was the more potent photosensitizer.

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V:I

MEDIUM-WAVE ULTRAVIOLET RADIATION (UVB) IS IMPORTANT IN
DOXYCYCLINE PHOTOTOXICITY

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ABSTRACT

The phototoxic reaction to doxycycline is provoked by long-wave ultraviolet light (UVA). It was shown by the in vivo mouse tail technique, measuring phototoxic edema, that the addition of medium-wave ultraviolet light (UVB) immediately after, immediately before and especially 24 h before the phototoxic trauma, enhanced the reaction more than could be expected from simple addition, thus demonstrating the importance of photoaugmentation in this process.

Key words: doxycycline
phototoxicity
mouse tail technique
photoaugmentation

INTRODUCTION

Doxycycline is a potent photosensitizer and the tetracycline derivative most frequently causing phototoxic reactions in Sweden as reported to the Drug Information Committee, Swedish National Board of Health and Welfare (1). The pronounced potency of doxycycline as well as demethylchlortetracycline (DMCT) and chlortetracycline compared to the other tetracycline derivatives has also been shown experimentally using red blood cells (2), lymphocytes (3) and fibroblasts (4) as well as in comparative studies in humans given tetracycline per os and exposed to natural sunlight (5,6,7). Doxycycline phototoxicity has also been demonstrated by in vivo experiments using the quantitative mouse tail technique although it should be

pointed out that the required dose was very high compared to other well known photosensitizers (8).

In spite of this mass of evidence it has been difficult to reproduce the phototoxic reaction in man using an artificial UVA source and doxycycline (orally) (9, Bjellerup and Ljunggren: unpublished results). The reason for this difficulty may be the lack of UVB in the artificial, as opposed to the natural situation, where UVA and UVB combine. The phenomenon of UVB photoaugmentation in drug phototoxicity has been shown earlier in mice for well known phototoxic drugs, especially chlorpromazine; tetracyclines however were not tested (10).

It therefore was found of interest to study the effect of UVB added to the phototoxic reaction elicited by doxycycline and UVA. The quantitative mouse tail technique (8) was found most convenient for the experiment.

MATERIAL AND METHODS

Animals

Female albino mice (AB Anticimex, Sollentuna, Sweden) weighing around 30 gm were used. The mouse tail technique, measuring phototoxic edema, has been described earlier (11).

Doxycycline

Doxycycline (provided by Pfizer, Brussels, Belgium) was dissolved in water and injected intraperitoneally at a concentration of 100 mg/kg bodyweight immediately before UVA irradiation.

Irradiation procedure

During exposure to ultraviolet light the animals were fixed in horizontal plastic tubes allowing only the tails to be exposed. The distance between the light source and the tails was 12 cm. The radiation output at the level of the tails was measured for UVA with a PUVA-meter and for UVB with a UV-meter (Waldmann AG, Schwenningen, GFR).

Light sources

For UVA 2 blacklight fluorescent tubes (Philips TLA 40W/08) with an emission peak at 360 nm were used. A 3 mm windowglass filter was inserted to eliminate wavelengths shorter than 320 nm, giving an output of 2.5 mW/cm² in the UVA region. For UVB 2 fluorescent tubes (FS 40 Westinghouse

Sun Lamp, 40 W) emitting continuously from 280-380nm with a peak at 313 nm were used, giving an output of 1.8 mW/cm^2 in the UVB region.

Experimental design

The effect of relatively small (in the mouse tail) doses of UVB given in connection with the phototoxic treatment (doxycycline + UVA) was studied in 6 experiments. According to fig. 1 UVB was given immediately before the phototoxic treatment in experiments no.1 and 2, immediately after in experiment no.3 and 24 hours before in experiments no.4, 5 and 6.

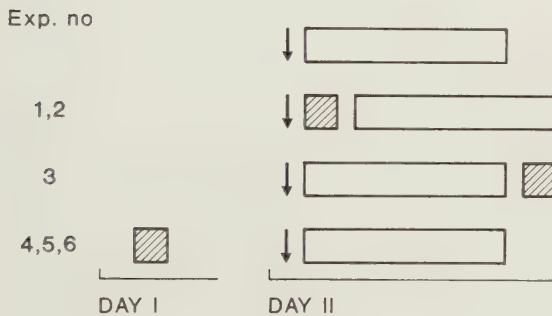


Fig 1. UVB given at different time intervals in connection with the phototoxic treatment (doxycycline + UVA) in the 6 experiments. UVA: open bars. UVB filled bars. Doxycycline injection: ↓ At the top the standard procedure of the quantitative mouse tail technique is shown i.e. no UVB is added.

To determine whether the addition of UVB caused an additive or augmentative effect 5 groups of 10 animals were needed in each experiment, treated according to table 1.

Thus group I was given UVA only, serving as a control since previous (10) and fresh pilot experiments had shown that this treatment induces no inflammatory reaction whatsoever. Group II was given UVB only, group III UVB and UVA, group IV doxycycline and UVA and group V UVB, doxycycline and UVA.

Exp. no. 1 and 2		Group no.				
		I	II	III	IV	V
day 1	Doxycycline				+	+
day 1	UVB (40, 20 min)		+	+		+
day 1	UVA (5 h)	+		+	+	+
Exp. no. 3		I	II	III	IV	V
day 1	Doxycycline				+	+
day 1	UVA (5 h)	+		+	+	+
day 1	UVB (40 min)		+	+		+
Exp. no. 4, 5 and 6		I	II	III	IV	V
day 1	UVB (40, 20, 10 min)		+	+		+
day 2	Doxycycline				+	+
day 2	UVA (5 h)	+		+	+	+

Table 1. The treatment of the 5 animal groups in each of the 6 experiments.

Evaluation and Statistics

The animals were sacrificed 24 h after the beginning of UVA exposure. A piece of the tail was excised, weighed, dried at 110°C and weighed again. Results are presented as percent wet weight increase over controls. To determine if the inflammatory response in group V (each animal was treated with the full combination UVB, doxycycline and UVA) was greater than expected from simple addition, i.e. augmentation had taken place, this group was compared to an addition of the control group given only UVA (group I), the UVB edema (wet weight % in group II minus I) and the phototoxic edema (wet weight % in group IV minus I), for graphic explanation see fig 2. The comparison of group V to the added values $[I + (IV - I) + (II - I)]$ was made using Student's t-test.

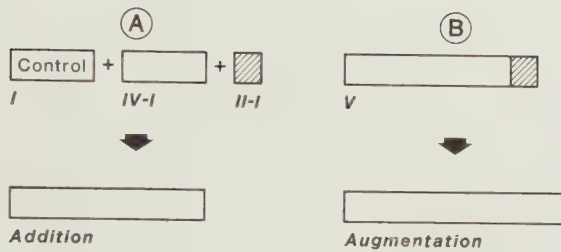


Fig 2. Comparison between addition of control group I wet weight, UVB-edema (filled bars) and phototoxic edema (open bars) given to separate groups of animals i.e. edema of group [I + (IV - I) + (II - I)][Ⓐ], compared to group V where all three components were given to one and the same animal[Ⓑ].

RESULTS

Exposure to UVB immediately before the phototoxic reaction

In experiments 1 and 2 UVB 4.3 J/cm^2 (40 min) and 2.2 J/cm^2 (20 min) respectively was given immediately before irradiating doxycycline treated animals with UVA 45 J/cm^2 (5 h). In experiment 1 the wet weight increase in group V was significantly higher than expected from addition (difference 3.7 percental units) (fig 3). In experiment 2 with the lower UVB dose there was a similar difference although not significant (1.9 percental units) (fig 3).

Exposure to UVB immediately after the phototoxic reaction

In experiment 3 UVB 4.3 J/cm^2 (40 min) was given immediately after UVA exposure of doxycycline treated animals. The wet weight increase in group V was significantly higher than expected (difference 4.3 percental units) (fig.3).

Exposure to UVB 24 h before the phototoxic reaction

In experiments 4, 5 and 6 UVB 4.3 J/cm^2 (40 min), 2.2 J/cm^2 (20 min) and 1.1 J/cm^2 (10 min) was given 24 h before doxycycline and UVA resulting in wet weight increases significantly higher than expected (difference 4.5, 10.3 and 7.5 percental units respectively) (fig 3).

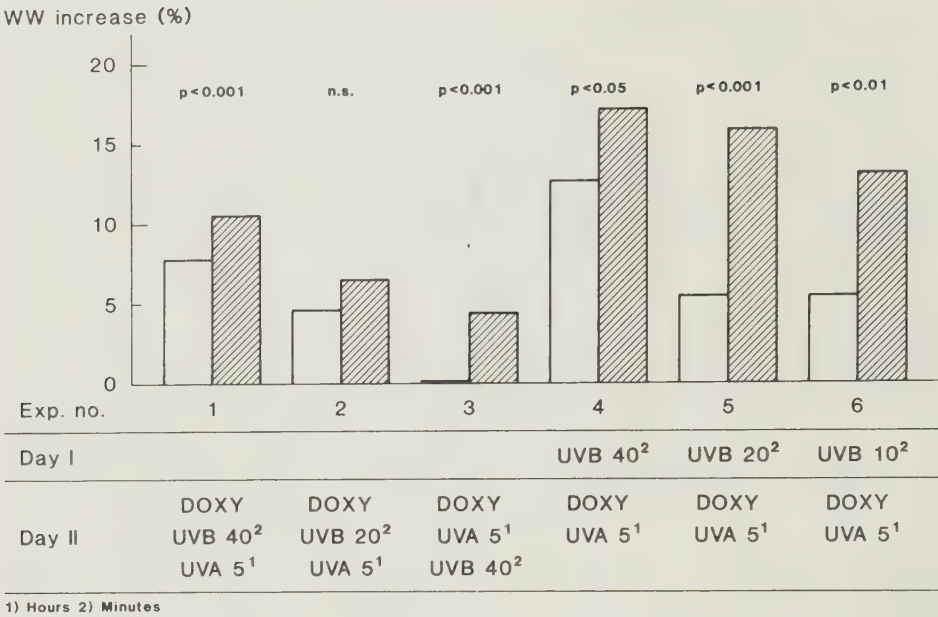


Fig. 3. Results of UVB-addition to the phototoxic reaction from doxycycline + UVA, testing the effect of varying UVB doses and different time intervals. For each of the 6 experiments a comparison between a calculated simple additive effect (open columns) and the *de facto* augmentative effect (black columns) is made. The height of the columns shows the wet weight increase over base line values. For explanation also see fig 2.

In none of the 6 experiments was there demonstrated any augmentation with the combination of UVA and UVB without doxycycline (group III, table 1).

DISCUSSION

The addition of small doses of UVB to the phototoxic doxycycline reaction thus increased the inflammatory mouse tail edema more than could be explained by simple addition, i.e., a photoaugmentation was demonstrated. The difference was statistically significant in all experiments but one (nr 2, fig 3) where the UVB dose evidently was too low to induce augmentation. When UVB was given in immediate connection with the phototoxic reaction the UVB/UVA order was of no importance (exp.no. 1, 2 and 3).

Photoaugmentation with UVB in drug phototoxicity, and the insignificance of UVB/UVA order, has been shown in mice earlier with chlorpromazine, chlordiazepoxide and 8-methoxypsoralen, however UVB was given only in immediate connection with UVA in these experiments (10).

In the present experiments UVB given 24 h before the phototoxic reaction resulted in an even stronger augmentation, maximally causing a percentual wet weight increase five times higher as when the same UVB dose was given in immediate connection with UVA (fig 3 experiments 2 and 5). A possible explanation for this is the increased concentration of tetracycline in inflamed skin as has been reported in man (12). In this situation the augmentation thus is proposed to be a combination of the general augmentative effect independent of UVB/UVA order, seen in the other experiments (no 1,2,3) and the concentration of drug in UVB-inflamed tissue.

It is thus concluded that UVB, although unable to induce tetracycline phototoxicity in itself, significantly modifies the phototoxic reaction from doxycycline and UVA in different ways, not only by addition but by augmentation. The lack of awareness of this phenomenon may be responsible for the difficulties in provoking tetracycline phototoxicity in humans using artificial light sources emitting in the UVA only (9, Bjellerup and Ljunggren, unpublished results).

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